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TITLE: Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

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## ABSTRACT

Prostate cancer is now the second highest cause of cancer death in men in Western society. New treatments are needed for late stage disease that has become refractory to hormone removal. We are using gene therapy, alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible organ, coupled with help from the immune system can promote reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5-FC), is then given, cancer cells that make CD/UPRT convert 5-FC to a toxin that kills the original cell and others nearby. This system works in slow growing s like prostate cancer. Killing the cells attracts immune cells. We are using the cytokines, Interleukin-12 or Interleukin-18 either alone or in combination, to upregulate the immune response against the cancer. We will deliver the cytokine gene alone or with the suicide gene because in other studies, combination therapy works better.

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# Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

## INTRODUCTION:

Prostate cancer is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible organ, coupled with help from the immune system can promote reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells that make CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This system works in slow growing s like prostate cancer. Killing the cancer cells attracts immune cells. The **scope** of the work involves identification of the immune cells that infiltrate the prostate when gene therapy is used. We will identify these cells and use cytokines to attract more of them to the site. We will then compare the effects of delivering the cytokine gene alone, the suicide gene alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of cancer cell deposits that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with prostate cancer.

## BODY:

New cell lines were prepared for our studies as described in our report in December, 2003. To test the proof of principle that a combination of gene therapy and immune therapy will target distant as well as local prostate cancer, we decided to use stably transfected cell lines derived from the mouse RM1 line, from T Thompson, Baylor College Texas, rather than using a viral delivery vehicle, thus avoiding any complications resulting from an immune response against the virus. As problems with intellectual property prevented us from continuing our work with the gene, purine nucleoside phosphorylase, for gene-directed enzyme prodrug therapy (GDEPT) directed against prostate cancer (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al.*, 2002), we have used the fusion gene, cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) as described in our December, 2003 report. CDUPRT has not previously been tested against prostate cancer, making this application novel. The prodrug is 5-fluorocytosine (5FC), which is converted by CD to 5-fluoro-uridine (5FU). The fusion gene has been shown to be more effective in GDEPT than CD alone, as the UPRT converts 5FU directly to additional anti- metabolites, 5FdUMP and 5FUTP (Tiraby *et al.*, 1998) and sensitises cancer cells to low doses of 5FU (Kanai *et al.*, 1998). The *drugs generated by CDUPRT can kill both dividing and non-dividing cells.* This is important in prostate cancer, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

We also decided that it would be preferable to use cells to carry the cytokine genes of interest into the mice, rather than using lipid-plasmid combinations, which would be less efficient. This would allow us to generate a maximum effect, and so achieve a proof of principle more quickly than by using lipid-based transfection *in vivo*.

**Trainee-Fellowship**: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. Dr Rosetta Martiniello-Wilks started on the program of work in February, 2003 (instead of September, 2002). However, she left the program in August, to take up a permanent position at another Hospital/ University in Sydney. She was replaced with Dr Bing Zhang, who started in January 2004, but left in June, 2004 because of unforeseeable family considerations. Natalia Liem continued this work for a few months, but also had to leave as she was pregnant and very sick with her pregnancy. We obtained permission from the DOD to employ a research assistant to complete the studies, but have not been able at this stage to find someone suitable. We will be seeking a no-cost extension for 6 months in order to complete the studies.

**Research/Training** Please note that Dr Martiniello-Wilks had not previously performed molecular biological work. She received training in the Oncology Research Centre, Prince of Wales Hospital, Department of Clinical Medicine, University of New South Wales; this work is described below.

**Reporting**: A report of our studies was provided in December 2003 and again in October, 2004.

Revised Statement of Work.
This was submitted previously. The tasks are outlined below:

**Revised Statement of Work**
**Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy 'Distant Bystander Effect' to Target Prostate Cancer Metastasis** ***(Amended 4th December 2002)***
DAMD17-01-1-0083

**Principal Investigator:** was Rosetta Martiniello-Wilks, PhD
*(to be appointed)*

*Task 1. Characterisation of the extent of the local bystander effect generated by* CD/UPRT-GDEPT

*(Months 1-6)*

a. Development of a stable *CD/UPRT*-RM-1 cell line
b. Produce cytokine plasmids, to express mIL-12, mIL-18 or a combination.
c. Assess the extent and duration of *CD/UPRT* expression in mouse *CD/UPRT*-RM1 prostate tumors by *in vitro CD/UPRT-GDEPT* assay
d. Assess the impact of the GDEPT local bystander effect on RM1 tumor growth mice

**Task 2. Characterisation the immune cells responsible for the GDEPT distant bystander effect and to assess their impact on local and remote site PCa growth (Months 6-14)**
a. *In vitro* studies will be performed to identify:
1. Treatment efficacy on prostate and lung tumor progression
2. Changes in immune cell recruitment to the prostate by histology

b. Immune cells that mediate the GDEPT distant bystander effect *in vivo* will be identified by chronic Mab depletion (3 groups of 30 mice [half treated with test Mab and the reminder with isotype control Mab], 4 test Mabs). These studies should provide the identity and timing of those cells mediating the distant bystander effect.

**Task 3. To assess the ability of locally delivered cytokine gene therapy to augment the GDEPT distant bystander effect (Months 13-20).**
a. Assess the tumor cytokine expression and secretion following treatment with doses of lipid-formulated cytokine plasmid delivery (lipid-pCytokine) (or adenovirus delivered cytokines) using RT-PCR, Western blot and biological activity assay (3 groups of 35 mice, 3 test pCytokines).
b. Assess the persistence of cytokine expression by tumors in vivo by Western blot (2 groups of 15 mice, 3 test pCytokines).
c. Assess the impact of local delivery of pCytokine treatment on CD/UPRT expression in tumors by in vitro CDUPRT assay (2 groups of 15 mice, 3 test pCytokines).
d. Assess augmentation of the distant bystander effect by local delivery of pCytokine treatment as described in Task 2a (2 groups of 15 mice, 3 test pCytokines)

**Task 4. To assess the efficay of GDEPT & cytokine gene therapy to suppress local/remote site PCas (Months 20-22)**
Treatment efficacy on prostate and lung tumor progression will be assessed. (3 groups of 30 mice).

**Task 5. Collate data, prepare reports and manuscripts (Months 22-24).**

**Reports of studies:**

**Task 1: (completed)**

**Characterization of the extent of the local bystander effect generated by CDUPRT –GDEPT**

This part of the work is covered by our publication: Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice by Aparajita Khatri, Bing Zhang, Eboney Doherty, Jane Chapman, Kim Ow, Hnin Pwint, Rosetta Martiniello-Wilks, Pamela J Russell, that has been submitted for publication to Molecular Therapy. A copy of the paper is attached in Appendix 1.

When examined *in vitro,* medium collected from RM1-CDUPRT/5FC but not from RM1-LacZ/5FC cells prevented the growth of RM1 cells ($P<0.0001$) indicating a CDUPRT-specific bystander effect (Khatri *et al*, Fig 3A). This was assessed *in vivo* by implanting mixtures of RM1-CDUPRT and RM1-GFP cells iprost in different proportions, followed by treatment with 5FC. Prostate tumor volumes measured at necroscopy indicated that the minimal proportion of RM1-CDUPRT cells required to produce a therapeutic effect was 20% ($P=0.01$) (Khatri *et al*, Figure 3B).

**Task 2:**

**Characterisation the immune cells responsible for the GDEPT distant bystander effect and to assess their impact on local and remote site PCa growth**

a. *In vivo* studies will be performed to identify:

1. Treatment efficacy on prostate and lung tumor progression **(completed)**

In the paper submitted, we investigated if CDUPRT GDEPT leads to a '*distant bystander effect*' by assessing whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of the parental RM1 cells in the lung. Three independent experiments were performed and data from a representative experiment is shown. The number of lung colonies in mice in the RM1-CDUPRT/5FC group was much lower than those in the control RM1-GFP/5FC or RM1-CDUPRT/saline groups (Khatri *et al*, Fig 4A and 4B). The lungs of the control groups were completely covered with RM1 tumors in all mice and this posed logistical problems. They were arbitrarily given the value of 450 colonies (Khatri *et al*, Fig 4B) on the basis of the average of counts done in 3 representative mice from each control group. While similar trends were seen in all 3 experiments, lung colony numbers varied between experiments. In one, 50% of the GDEPT treated mice had no lung colonies; there were no such mice in control groups (data not shown). This suggests that a '*distant bystander effect*' may have prevented the growth of RM1 lung colonies.

2. Changes in immune cell recruitment to the prostate by histology **(completed)**

As described in the paper submitted under Task 1 by Khatri *et al*, cells infiltrating the prostate after GDEPT were examined by immunohistochemistry. The distant bystander effect is characterized by infiltration by immune cells e.g. macrophages, $CD8^+$ $CD4^+$ T, B and NK cells in tumors undergoing GDEPT (21-25). Immunoperoxidase staining was used to assess infiltration by macrophages, $CD8^+$ $CD4^+$ T, and NK cells in Iprost tumors from different treatment groups (Khatri *et al*, Table 2). Increasing numbers of $CD4^+$ T cells were detected in all three RM1-CDPURT+5FC treatment groups (B, C and D) compared with RM1-GFP+5FC control group (A) ($P=0.003$). $CD4^+$ T cell recruitment was the most significant (7x) in tumors from 100% RM1-CDPURT/5FC group compared with 2x in 20% and 10% mixed cell tumors (2x) (Khatri *et al*, Fig 6A). In contrast, staining for $CD8a^+$ cells was minimal in all groups. Although not statistically significant, increasing numbers of infiltrating $F4/80^+$ (macrophages) and Asialo-$GM1^+$ (NK) cells were seen in tumors of the three RM1-CDPURT+5FC groups (Khatri *et al*, Figs 6B, 6C) compared with the control group (Khatri *et al,* Fig 6A; Table 2); representative sections from treated tumors are shown (Khatri *et al*, Fig. 6). This CDUPRT-specific dose dependant increase in $CD4^+$, macrophages and NK cells is a strong indication of the involvement of the immune system in local and distant bystander effects.

Task 2b. Immune cells that mediate the GDEPT distant bystander effect *in vivo* will be identified by chronic Mab depletion (3 groups of 30 mice [half treated with test Mab and the reminder with isotype control Mab], 4 test Mabs). These studies should provide the identity and timing of those cells mediating the distant bystander effect.
**This work has not yet been performed.**

**Task 3. To assess the ability of locally delivered cytokine gene therapy to augment the GDEPT distant bystander effect**
**Task 4. To assess the efficacy of trimodal GDEPT & cytokine gene therapy to suppress local/remote site PCas**
Given the difficulties we have had because of changing staff, we designed an experiment to test tasks 3 and 4 as follows: At this stage, we have done this work once, and repeated aspects of it.
Experimental Design:
In general, mice implanted with Iprost RM1CDUPRT tumors were given intratumoral (i.t.) injections of either pVITRO2GFP.mIL12 or pVITRO2GFP.mIL18 or a combination of the two with and without 5FC. The plasmid pVITRO2GFP.LacZ was used as the control plasmid and RM1GFP.LacZ cells were used as control cells for these experiments. The experiment was performed as follows:
Day 0: Mice were implanted intraprostatically with 2.5 x$10^4$ RM1CDUPRT cells or 5 x $10^4$ RM1LacZ cells.
Day 6: Mice were given i.t. injections of either pVITRO2GFP.mIL12/ pVITRO2GFP.LacZ or pVITRO2GFP.mIL18/ pVITRO2GFP.LacZ or a combination of the two, pVITRO2GFP.mIL12/ pVITRO2GFP.mIL18 at the dose of 19μg of each plasmid /mouse.
Day 7: Mice were injected with 2.5 x$10^5$ RM1 parental cells intravenously to establish psuedometastases. Mice were also given 5FC (500 mg/kg/mouse/day) or saline, intraperitoneally until necroscopy (day 16).
At necroscopy, mice were euthanased and tumors and other organs such as spleen and draining lymph nodes were harvested and were either formalin fixed or snap frozen (Liquid Nitrogen) for histological and immunohistogical analyses. Mouse serum was also harvested and stored at –80°C for future analyses. The lungs were fixed in Bouin's reagent and colonies were counted as described in Khatri *et al.*, 2005.

***The experiment was done in two parts due to logistical problems associated with large numbers of mice involved.***

Experiment 1: To evaluate the efficacy of CDUPRT GDEPT in combination with either pVITRO2GFP.mIL12 or pVITRO2GFP.mIL18.
The treatment groups were as follows:

1. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @19μg/plasmid/mouse (total 38μg/mouse) with saline (ip,everyday until necroscopy).
2. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @19μg/plasmid/mouse with 5FC (ip,everyday until necroscopy).
3. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @19μg/plasmid/mouse with saline (ip,everyday until necroscopy)
4. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @19μg/plasmid/mouse with 5FC(ip,everyday until necroscopy)
5. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.LacZ @38μg/plasmid/mouse with saline (ip, everyday until necroscopy)

6. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.LacZ @38μg/plasmid/mouse with 5FC (ip,everyday until necroscopy)

Results:

The growth of RM1CDUPRT and RM1 LacZ tumours was reassessed in the prostate of C57BL/6 mice because the cell lines had to be rederived *in vivo*, due to possible Mycoplasma contamination. Both cell lines grew in a dose dependent manner, however the RM1CDUPRT cells grew more rapidly compared with the control cell line, RM1LacZ (Figure 1). On the basis of these results, the dose of cells for iprost injection chosen was $2.5x10^4$ cells for RM1CDUPRT and $5x10^4$ for RM1LacZ cells.

Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 or pVITRO2.mIL18 on growth of intraprostatic RM1 tumors in C57BL/6 mice

There were no effects of any treatment on the growth of iprost tumors at necroscopy of the different groups of mice (Figure 2). This result was surprising, especially given that CDUPRT/5FC GDEPT had previously been shown to diminish prostate tumor growth (see paper, Appendix 1). A possible explanation is that the RM1CDUPRT cells were implanted at five fold the doses that we used earlier, based on the growth of the rederived cells (Figure 1). Since the tumors grew to very large volumes, the aggressive growth of the RM1CDUPRT tumors may have overcome the effects of therapy in the timeframe involved. We intend to repeat this experiment using a lower dose of RM1CDUPRT cells.

Distant bystander effects

RM1 tumor pseudometastases in lungs were assessed. In contrast to the tumor growth in the prostate, the growth of RM1 lung colonies was GDEPT (CDUPRT/5FC) dependent in all treatment groups (Figure 3). However, neither pVITRO2.mIL12 nor pVITRO2.mIL18 showed any additive or synergistic effects with CDUPRT/5FC GDEPT.

Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 and pVITRO2.mIL18 on growth of intraprostatic RM1 tumors in C57BL/6 mice

As with the use of either plasmid alone, a combination of pVITRO2.mIL12 and –mIL18 with CDUPRT/5FC GDEPT had no significant effect on the growth of tumors in the prostate (Figure 4). Again, we believe that any effects may have been masked by the aggressive growth of the tumors, and will be repeating this experiment with a lower dose of RM1CDUPRT cells.

Distant bystander effects

In contrast to the effects of each plasmid alone, the combination of pVITRO2.mIL12 and -mIL18 was extremely potent and dramatically diminished the lung colony formation, irrespective of the GDEPT (Figure 5). Whilst the local effects of either plasmid or a combination of the two were absent, the distant bystander effects indicated that the treatments were effective, most likely through stimulation of an immune response.

Studies of immune response.

Prostates were harvested at necropsy from each treatment group and snap frozen sections from 3 mice/group were immunostained for the presence of CD4+ and CD8+ T cells, macrophages and NK cells. These preliminary data are summarised in Table 1; more mice will be examined from each group to generate statistically significant results. There were several points of interest:

1. CD4 staining: Combining mIL18 + GDEPT (C) resulted in increased CD4+ T cells in the tumor compared to mIL12 + GDEPT (B) or GDEPT alone (A) (Figure 6). Using both plasmids together caused significantly increased (3.5-fold) CD4+ cells with or without GDEPT and when RM1LacZ cells were used in combination with mIL12 + mIL18 (E), the increase in CD4+ cells was higher than that seen with RM1CDUPRT cells (D). This effect was more acute when 5FC was also used. No significant differences were seen between saline and 5FC groups in all treatment groups. This finding suggests that CD4+ T cells are involved in the distant bystander effect seen when mIL12 and mIL18 were combined.

2. CD8A staining: Increased CD8+ cells were seen in tumors treated with mIL18 plasmid alone (C), and in those that received mIL18+ mIL12 (D and E), but not when mIL12 (B) or GDEPT (A) were used alone. There was a trend to enhancement of CD8+ T cell infiltration in the presence of GDEPT with 5FC in C, D and E compared to saline. This increase was the most significant in E, suggesting greater immunogenicity of RM1LacZ cells compared with RM1CDUPRT cells, possibly due to a greater immune response to the LacZ gene product.
3. F4/80 staining: In contrast to the trends observed with CD4+ and CD8+ infiltration, macrophage infiltration was minimal in tumors treated with mIL18 plasmid (C) compared to those treated with mIL12 plasmid (A) or GDEPT alone (B). The presence of CDUPRT/5FC GDEPT appeared to diminish the macrophage infiltration (A-D) compared to tumors from RM1LacZ cells (E). This may reflect the immunosuppressive nature of 5FU generation by the GDEPT. Infiltration was significant when RM1Lac Z cells were used with the two plasmids (E) (Figure 7), or RM1CDUPRT cells were injected with pVITRO2.LacZ (A), again supporting our hypothesis that the LacZ gene product may be immunogenic.
4. Asialo GM: There were no significant differences between NK cell infiltration seen in all treatment groups (Figure 8). However a general reduction was observed in 5FC treated groups compared with saline treated controls in treatment groups A,C and D (Figure 9). The use of mIL18 plasmid alone + GDEPT (C) appeared to suppress asialo GM+ cell infiltration, but not when used in combination with mIL12 plasmid + GDEPT and RM1LacZ cells (E). When pVITRO2.mIL12 was used alone with GDEPT, there was no reduction in asialoGM+ cells, suggesting that the effects of mIL12 on NK cell infiltration are more potent than those of mIL18 alone in overcoming any immunosuppression generated by the GDEPT.

Table 1: Immunohistochemical analysis of immune cell infiltration in intraprostate tumors from different treatment groups

| | *CD4*$^+$ | | *CD8*$^+$ | | **F4/80**$^+$ | | **AsialoGM**$^+$ | |
|---|---|---|---|---|---|---|---|---|
| | ***Saline*** | ***5FC*** | ***Saline*** | ***5FC*** | ***Saline*** | ***5FC*** | ***Saline*** | ***5FC*** |
| **A:RM1-CDUPRT+pvitro2GFPLacZ** | 6.7 | 1.1 | 2.4 | 1.2 | 23.5 | 8.8 | 26.6 | 14.1 |
| **B:RM1-CDUPRT+pvitro2GFPLacZ+pvitro2 GFPmIL12** | 1.6 | 1 | 2.7 | 1 | 15.2 | 11.6 | 21.4 | 21.1 |
| **C:RM1-CDUPRT+pvitro2GFPLacZ+pvitro2 GFPmIL18** | 8.3 | 10.2 | 2.5 | 6.8 | 7.8 | 1 | 21.6 | 5.4 |
| **D:RM1-CDUPRT+pvitro2GFPmIL12+pvitro2 GFPmIL18** | 18.7 | 10.6 | 8.6 | 9.4 | 24.2 | 4.9 | 22.9 | 11.1 |
| **E:RM1-LacZ+pvitro2GFPmIL12+pvitro2GFP mIL18** | 22.2 | 25.2 | 7.6 | 16.2 | 33.1 | 28.4 | 22.5 | 20.1 |

In conclusion, we have shown that the combination of mIL12 and mIL18 plasmids have synergistic immunostimulatory effects in preventing pseudometastatic growth in our model. The lack of efficacy against the local intraprostatic tumor growth may be overcome by using a lower dose of these aggressive RM1CDUPRT cells for implantation (currently underway). Next, we will characterise the immune response generated by immunodepletion of CD4+, CD8+, and NK cells and macrophages *in vivo*.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP/mIL-12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.
- Established local and distant bystander effects of CDUPRT-GDEPT system in RM1 tumors *in vivo*.
- Measured functionality of expression of the IL-12 and IL-18 transgenes against CTLL2 cells
- Performed preliminary work to determine the growth rate of RM1-GFP/IL12 and RM1-GFP/IL18 lines grown subcutaneously or in the prostate of C57BL/6 mice *in vivo*.
- Established the distant bystander effects of CDUPRT-GDEPT and of trimodal therapy.

**REPORTABLE OUTCOMES:**

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL-12; RM1-GFP/mIL-18.
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.
- We have submitted a paper on the bystander effects seen when CDUPRT cells are injected into the prostate of C57BL/6 mice to Molecular Therapy (see appendix 1).
- We are currently writing a technical paper about the use of CTLL2 cells for examining the effects of mIL12 and mIL18 and the synergy between these two cytokines.
- Abstracts were presented at AACR and Australasian Gene Therapy Society conferences in 2005 (see appendix 3).

**CONCLUSIONS**

We have shown that gene directed enzyme prodrug therapy directed by CDUPRT plus 5FC can eliminate the growth of RM1 prostate cancer cells in the prostate of C57BL/6 mice, and is associated with an excellent local bystander effect, both *in vitro* and *in vivo*, as well as a distant bystander effect. Thus local therapy given into the prostate inhibits the growth of pseudometastases induced by the intravenous administration of RM1 cells.

We have also shown that mIL12 and mIL18 given alone can inhibit the growth of RM1 cells implanted subcutaneously or in the prostate of C57BL/6 mice.

We have now shown the synergistic effects of mIL12 and mIL18 against lung pseudometastases from RM1 cells *in vivo*. We have preliminary evidence that this is mediated by stimulation of CD4+, CD8+ T cells.

We have established a new model for examining the effects of mIL12 and mIL18 against CTLL2 cells grown *in vitro*, obviating the need to assess their functionality by *in vivo* experiments. We have demonstrated synergy between these two cytokines in their anti-proliferative effects against CTLL2 cells *in vitro*.

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**Appendix 1**
Paper submitted to Molecular Therapy: Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice by Aparajita Khatri, Bing Zhang, Eboney Doherty, Jane Chapman, Kim Ow, Hnin Pwint, Rosetta Martiniello-Wilks, Pamela J Russell

**Appendix 2**
Figures 1-9

**Appendix 3**
Abstracts presented:

# APPENDIX 1

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Title: Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice.

Article Type: Regular Article

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**Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate-cancer in mice.**

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**Running Title: CDUPRT-GDEPT using the mouse RM1 prostate cancer model**

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Abbreviations Used: AR, Androgen refractory; AS, Androgen sensitive; PC, Cancer of the prostate; FBS, Fetal bovine serum; GDEPT, Gene directed enzyme prodrug therapy; HSVtk, Thymidine kinase gene from Herpes Simplex Virus; PNP, purine nucleoside phosphorylase. HRPC, Hormone refractory prostate cancer.CD, cytosine deaminase; UPRT, uracil phosphoribosyl transferase; CDUPRT, cytosine deaminase in combination with uracil phosphoribosyl transferase. 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; GFP, Green Fluorescent protein; HPLC, High performance liquid chromatography; 5FdUMP, 5-fluoro-2'-deoxyuridine 5'monophosphate; 5FUTP, 5-fluorouridine 5'-triphosphate; 5FUMP, 5-fluorouridinemono-phosphate; ICGJ, Intercellular gap junctions; NK cells, Natural killer cells.

## ABSTRACT

We aimed to evaluate the efficacy of gene–directed enzyme prodrug therapy (GDEPT) using cytosine deaminase in combination with uracil phosphoribosyl transferase (CDUPRT) against intra-prostatic mouse androgen-refractory prostate (RM1) tumors in immunocompetent mice. The product of the fusion gene, CDUPRT, converts the prodrug, 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) and other cytotoxic metabolites that kill both CDUPRT-expressing and surrounding cells, via a 'bystander effect'. RM1 cells were stably transfected with plasmids containing green fluorescence protein (GFP)/CDUPRT, GFP or GFP/LacZ genes. CDUPRT expression in RM1-GFP/CDUPRT cells or tumors was confirmed by enzymic conversion of 5FC to 5FU, using HPLC. Treatment of mice bearing intra-prostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. A 'local bystander effect' was seen, even though only 20% of the cells expressed CDUPRT. More importantly a significant reduction in pseudo-metastases of RM1 cells in lungs indicated a 'distant bystander effect'. Immunohistochemical evaluation of the treated tumours showed increased necrosis and apoptosis, with decreased tumor vascularity. There was also a significant increase in tumour-infiltration by macrophages, $CD4^+$ T and natural killer cells. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate-tumors and lung pseudo-metastases via immune mechanisms involving necrosis and apoptosis.

**KEYWORDS:** GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

## INTRODUCTION

Of several GDEPT systems under investigation for PC (1), we are evaluating a fusion gene constructed by combining cytosine deaminase (CD) with uracil phosphoribosyltransferase (UPRT). Cytosine Deaminase is an enzyme of bacterial or fungal origin that converts the non-toxic pro-drug, 5 fluorocytosine (5FC) to 5-fluorouracil (5FU). 5FU is further modified by cellular enzymes to pyrimidine antimetabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) that inhibit DNA and RNA synthesis. Thus, 5FU should be effective in killing both dividing and non-dividing cells, an important factor, given the low percentage (2%) of dividing cells in PC (2). Despite the success of 5FU-chemotherapy for gastrointestinal, head and neck malignancies, it has shown a poor therapeutic index for other cancers primarily because some tumor cells develop immunity to 5FU toxicity. A major factor responsible is the low efficiency of conversion of 5FU into its toxic metabolites; the rate-limiting step is the formation of an intermediary metabolite, 5-fluorouridinemono-phosphate (5FUMP) involving a series of enzyme-catalyzed reactions (3). This is circumvented by the ability of UPRT to convert 5FU directly to 5FUMP leading to more efficient production of toxic metabolites (4) and hence increased sensitization (up to 15 fold) of the UPRT-transduced cells to 5FU (5). Use of UPRT in conjunction with CD sensitizes cancer cells to low doses of 5FU and 5FC (3,6,7) and results in greater anti-tumor efficacy compared with CD-GDEPT alone (7,8). Only limited studies have investigated the effects of CDUPRT GDEPT *in vivo* against PC.
Given that a 100% gene transfer efficiency with currently used gene delivery technology is unattainable *in vivo*, an important advantage of GDEPT is derived from the local bystander effect engendered; comprehensive cell killing is achieved without the need to express the gene in all cells (9,10). The local bystander effects associated with 5FC/5FU-based GDEPT is due to the ability of 5FU to freely diffuse between cells (7,11). However, the metabolites of 5FU can only

move through gap junctions (12) and therefore the extent of the bystander effects relies on the incidence of gap junctions in the cells. While the combination CDUPRT-GDEPT clearly results in a more efficient conversion of 5FU to its toxic metabolites, it may also lead to depletion of the cytoplasmic pool of freely diffusible 5FU that could compromise the bystander effect. Clearly, this needs to be investigated individually for different cell types. Although *in vitro* studies have shown that the local bystander effect associated with CDUPRT-GDEPT varies depending upon the cancer cell properties (7,11,13), none have evaluated the bystander effects against PC *in vivo*. Hence, in this study we sought to assess the local bystander effects of CDUPRT *in vitro* and *in vivo* against murine PC. Some GDEPTs including CD- and UPRT- lead to tumor cell killing in the remote locations, culminating in a *distant bystander effect* (1,14,15), which is mediated via treatment specific anti-tumor immune responses. To date there are no reports of any preclinical studies evaluating the distant bystander effects generated using the fusion gene, CDUPRT, so, we assessed its ability to produce a similar '*distant bystander effect*'.

An immunocompetent mouse model of syngeneic PC was used to examine the ability of locally given GDEPT to exert local and distant bystander effects. An androgen-refractory PC cell line (RM1) (16) was implanted into the prostate or tail vein of C57BL/6 mice leading to tumor formation and experimental lung metastasis, respectively, in an aggressive and reproducible manner (17).

RESULTS

**1. Efficacy of CDUPRT-GDEPT *in vivo***

*Optimization of 5FC dose:* An important feature of CDUPRT GDEPT is that both 5FC and 5FU (FDA approved drugs) can be used as prodrugs for this system. We chose 5FC as it is efficiently metabolized by the fusion gene and is non-toxic at the therapeutic doses used. We first determined the maximum non-toxic dose of prodrug usable in our model. C57BL/6 mice could tolerate 5FC at doses up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 14 days without systemic toxicity as shown by examination of Hematoxylin and Eosin (H&E) stained major organs (Fig.1A) and by serum analysis for biochemical markers of kidney and liver function (Urea Creatinine, ALP, ALT and AST (Fig.1B). Neither serum markers, nor histology differed between 5FC-injected versus control mice, suggesting that there was no detectable toxicity from 5FC even at the highest dose tested. There was no loss of weight or condition in the treated mice.

*Generation of stably transformed RM1 cell lines expressing GFP, GFP CDUPRT or GFPLacZ:* RM1 cells were stably transformed to express GFPCDUPRT, GFP or GFPLacZ (RM1GFP, -CDUPRT or -LacZ) and gene expression of the transgenes was established. For CDUPRT, *in vitro* RM1-CDUPRT cultures and tumors harvested after subcutaneous (sc) and intraprostatic (Iprost) growth in C57BL/6 mice were shown by HPLC to have the capacity to convert 5FC to 5FU, confirming CDUPRT expression and importantly, there was no loss of activity *in vivo* over the duration of the experiments (data not shown). GFP expression in all cell lines was confirmed by UV microscopy and flow cytometry.

*Evaluation of RM1 cells for assessment of CDUPRT GDEPT:* Given that some cells are resistant to 5FU therapy, toxicity of 5FU to RM1 cells was assessed *in vitro* to evaluate the suitability of our model for evaluating *CDUPRT GDEPT.* Cultured RM1 cells were subjected to different doses of 5FU for 1 week (data not shown) and then assessed for viability by cell counting via trypan blue exclusion. There was almost complete eradication of cells at dose > 10μg/mL (77μM, 85% cell death) at 72 h post treatment. Cytotoxic effects were seen (~57% cell death compared with untreated controls) even at 1μg/mL (7.7μM) by day 7, which was lower than that for the human PC DU145 cells (17μM) (13) but higher than the acceptable therapeutic range of 5FU in humans (1.9 ± 0.3μM). Although, doses lower than 7.7μM were not tested, these data suggested that RM1 cells are moderately sensitive to 5FU toxicity. When tested for 5FC sensitivity, control

RM1-LacZ cells were resistant up to the highest dose tested (100μg/ml) but RM1-CDUPRT cells were susceptible to cell killing even at 3μg/ml showing ~34 fold enhancement of 5FC sensitisation of RM1–CDUPRT cells (data not shown). This clearly established the suitability of our model for assessing CDUPRT-GDEPT.

*GDEPT using RM1CDUPRT/5FC:* To establish that RM1-CDUPRT cells could effect GDEPT *in vivo* in the presence of 5FC, mice were implanted Iprost with RM1CDUPRT or RM1-LacZ tumors and injected ip with 5FC or saline. GDEPT in the RM1-CDUPRT/5FC group was very effective with almost complete absence of growth in the prostate compared to mice in control groups (RM1-LacZ/5FC, RM1-CDUPRT/saline) (Fig.2A)(P=0.009). Histology of RM1-CDUPRT tumors from mice treated with saline (6 mice) or 5FC (10 mice) showed a highly vascularized and viable tumor (Fig 2B) in the former, whereas treatment with 5FC resulted in necrosis (Fig 2C), with loss of prostate tissue architecture (Fig 2C, inset). All tumors from CDUPRT/saline mice showed >80% viability, with <10% tumor necrosis or haemorrhagic necrosis (necrosis due to disruption of vasculature). In contrast, mice given 5FC had no tumors (in 6 cases), or <10% viable tumor (4 cases) with >30% necrosis, and >60% haemorrhagic necrosis in the latter (Table 1). Other tissues examined histologically (kidney, lung, spleen, liver), showed no abnormalities (data not shown), indicating the tumor-specific nature of the cytotoxic effects of the treatment with no apparent systemic toxicity. Finally, HPLC analysis of sera from mice from RM1-CDUPRT/5FC and RM1-LacZ/5FC groups showed no detectable levels of 5FC and 5FU in either (data not shown).

**2. Local bystander effect on RM1 growth**

Once the efficacy of the CDUPRT GDEPT was established, any local bystander effects resulting from the treatment were assessed *in vitro* and *in vivo*.

When examined *in vitro,* medium collected from RM1-CDUPRT/5FC but not from RM1-LacZ/5FC cells prevented the growth of RM1 cells ($p<0.0001$) indicating a CDUPRT-specific bystander effect (Fig 3A). This was assessed *in vivo* by implanting mixtures of RM1-CDUPRT and RM1-GFP cells Iprost in different proportions, followed by treatment with 5FC. Prostate tumor volumes measured at necroscopy indicated that the minimal proportion of RM1-CDUPRT cells required to produce a therapeutic effect was 20% ($p=0.01$) (Figure 3B).

*Apoptosis after GDEPT:* To investigate how GDEPT mediates cell death *in vivo*, the extent of apoptosis was evaluated by TUNEL assay (34) on tumors from different treatment groups (Fig 4A, Table2). Scattered apoptotic cells were seen throughout the tumors of RM1-LacZ/5FC or RM1-CDUPRT/saline controls. Apoptosis was markedly increased in tumors of the GDEPT groups in a dose dependant manner. An increase of 1.8 fold was seen in 100% RM1-CDUPRT/5FC group compared with 100%RM1-GFP/5FC group ($p=0.0001$), in both necrotic and non-necrotic areas.

*Tumor vasculature after GDEPT:* We noted in the previous experiment that CDUPRT-GDEPT tumors were characterised by extensive haemorrhagic necrosis suggesting that the treatment may have disrupted the tumor vasculature. To investigate this possibility, vascular analysis (anti-CD31 staining) of the entire tumor section was performed. Any stained endothelial cells or clusters separated from adjacent microvessels were included and counted as one microvessel, whereas infrequent CD31-positive macrophages and plasma cells were excluded from the analysis. Neither vessel lumens nor red blood cells were used to define a microvessel (18). CDUPRT/FC caused a reduction in the vascularity of the tumors by more than 3X compared to the control RM1-GFP tumors (Table 2, Fig. 4B, $p=0.006$). This extent of reduction increased with increasing number of CDUPRT expressing cells (Table 2) suggesting the vasculature disruption to be CDUPRT-GDEPT-specific and possibly involved in the enhancement of its cytotoxicity.

**3. Distant bystander effects of CDUPRT-GDEPT**

Next, we investigated if CDUPRT GDEPT leads to a similar *'distant bystander effect'* by assessment of whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of the parental RM1 cells in the lung. Three independent experiments were performed and data from a representative experiment is shown. The number of lung colonies in mice in the RM1-CDUPRT/5FC group was much lower than those in the control RM1-GFP/5FC or RM1-CDUPRT/saline groups (Fig.4A and 4B). The lungs of the control groups were completely covered with RM1 tumors in all mice and this posed logistical problems. Therefore, they were arbitrarily given the value of 450 colonies (Fig. 4B) on the basis of the average of counts done in 3 representative mice from each control group. While similar trends were seen in all three experiments, there were variations in lung colony numbers between experiments. In one experiment, 50% of the GDEPT treated mice had no lung colonies compared with no such mice in the control groups (data not shown). This suggests that a *'distant bystander effect'* may have prevented the growth of RM1 lung colonies.

***Immunohistochemical studies of RM1 tumors*** The distant bystander effect is characterized by infiltration by the immune cells e.g. macrophages, $CD8^+$ $CD4^+$ T, B and NK cells in tumors undergoing GDEPT (19-22). Immunoperoxidase staining was used to assess infiltration by macrophages, $CD8^+$ $CD4^+$ T, and NK cells in Iprost tumors from different treatment groups (Table 2). Increasing numbers of $CD4^+$ T cells were detected in all three RM1-CDPURT/5FC treatment groups (B, C and D) compared with RM1-GFP+5FC control group (A) ($p<0.05$). Further, this recruitment was enhanced by 7X in tumors from 100% RM1-CDPURT/5FC group compared with 2X in 20% and 10% mixed cell tumors (Fig 6A). In contrast, staining for $CD8a^+$ cells was minimal in all groups. Although not statistically significant, increasing numbers of infiltrating $F4/80^+$ (macrophages) and Asialo-$GM1^+$ (including NK) cells were detected in tumors of the three RM1-CDPURT+5FC groups (B, C) compared with the control group (A, Table 2); representative sections from treated tumors are shown in Fig. 6. This CDUPRT-specific dose dependant increase in $CD4^+$, macrophages and NK cells is a strong indication of the involvement of the immune system in local and distant bystander effects.

**DISCUSSION**

We previously reported that a single dose of PNP-GDEPT was effective against orthotopic RM1 tumors, leading to increased survival (17,23). We have now explored a new GDEPT system, CDUPRT fusion gene with the prodrug, 5FC. We wanted to examine the local and distant bystander effects of CDUPRT-GDEPT in the context of an intact immune system particularly as it was implicated in developing systemic anti-tumorigenicity with CD/5FC and UPRT/5FU GDEPTs (24,25). We used a syngeneic immunocompetent mouse model of orthotopic and pseudo-metastatic murine PC (26) which was further characterized by us (27). This model is susceptible to other GDEPTs (HSV/TK, PNP) (17,23,28). Further, RM1 cells are mildly immunogenic and express MHC class 1 molecules making them susceptible to immune system mediated cell killing (28,29). Further when tested for sensitivity to 5FU, sensitivity of RM1 cells (57% cell killing at 1μg/mL (7.7μM) was comparable to that of mouse mammary carcinoma (1μM) and mouse lymphoma cells (10μM) (30). This was higher than the accepted therapeutic range in humans and offered an ideal therapeutic window for assessment of CDUPRT GDEPT. When tested for 5FC sensitivity, the control RM1LacZ cells were not affected even at 100μg/mL of 5FC but efficient cell killing was seen for RM1-CDUPRT cells at low doses of >3μg/mL, well within the accepted steady state levels in humans (50μg/ml) (31). The 5FC related systemic toxicities were undetectable in mice with no loss of condition at the experimental dose (500 mg/kg/mouse) commonly used in other studies (6).

The *in vivo* efficacy of the CDUPRT GDEPT against RM1 Iprost tumors was unequivocally proven and supports other studies showing the efficacy of the combination against different types of cancers including PC (6,7,13). Treatment was associated with a local bystander effect (Fig 3), and stimulated a distant bystander effect as evidenced by inhibition of pseudo-metastases in lungs (Fig 5). *This is the first in vivo demonstration of local and distant bystander effects in PC using the fusion gene, CDUPRT.* Bystander effects of CDUPRT GDEPT are operated via two independent mechanisms: diffusion of cytotoxic 5FU and transfer of the cytotoxic fluoronucleotides (FUMP and FUTP) via intercellular gap junctions (ICGJ) to surrounding cells. This provides for the cell type-based variation in the bystander effects. Indeed, *in vitro* local bystander effects caused by CDUPRT-GDEPT are described for glioma (6), glioblastoma (11) and colon carcinoma (7), generally when 2- 10% of cells expressed the transgene. This variability was due to differences in 5FU sensitivity (11) and ICGJ status of the cells (6). Miyagi *et al* (13) showed that ICGJ lacking DU145 human PC cells showed only 30% cell killing when 10% cells were expressing CDUPRT, *in vitro.* While CDUPRT- GDEPT was more effective than CD-GDEPT against DU145 xenografts, the local bystander effects for these two GDEPTs against DU145 cells showed an opposite trend *in vitro.* The authors postulated that although the active phosphorylated metabolites of the CDUPRT GDEPT could not diffuse efficiently in DU145 cells which lack ICGJ (12), a more efficient conversion of 5FC into 5FU sensitized the cells to 5FU leading to enhanced anti-tumor killing *in vivo.*

In our study, we demonstrated the presence of the local bystander effect *in vitro* qualitatively. Use of cell mixtures containing different proportions of RM1 cells with RM1-GFP-CDUPRT cells did not yield significant data as the RM1 cells grow twice as fast compared with RM1CDUPRT cells both *in vitro* and *in vivo* (data not shown). Interestingly, when investigated *in vivo,* the data clearly showed a bystander effect when 20% of the cells expressed the transgene. This was significant given that the faster growth rate of RM1 cells would bias the results against the demonstration of the bystander effects. Other factors that could affect the extent of bystander effect in our system: A. Moderate sensitivity of the RM1 cells to 5FU toxicity (7.7 μM compared with 17μM in 5FU resistant DU145 cells) B. Diminution of the pool of available 5FU due to efficient conversion to its metabolites (4) C. RM1 cells lead to aggressive non-differentiated tumors *in vivo* (27) and it is known that loss of ICGJ is a critical step in progression to human prostate neoplasia (32). It is likely that intercellular gap junctions are absent or present at low concentration in RM1 tumors and hence the bystander effects are not as dramatic as expected from other studies (6,7).

*Our laboratory is the first to describe the distant bystander effect of CDUPRT-GDEPT in vivo.* We have shown that operating the CDUPRT-GDEPT in the prostate of mice results in a considerable suppression of parental RM1 colony formation in the lungs.

Two factors may contribute to this. The first is due to chemically induced responses related to RM1-sensitivity to systemically given 5FC or 5FU introduced into the bloodstream during GDEPT. It was unlikely that 5FC contributed to toxicity as RM1 cells were resistant to 5FC even at 100μg/mL and serum and histological analysis of organs from mice given 5FC did not show any 5FC related toxicity; also 5FC or 5FU could not be detected in the blood sera of the treated mice. This suggested an alternate mechanism such as anti-tumor immune responses triggered during GDEPT may have mediated cell killing of RM1 cells. It was postulated (33) that a distant bystander effect is induced due to release of cytokines via immune cell activation, triggered by GDEPT induced tumor destruction. This leads to hemorrhagic necrosis that then allows more immune cells to infiltrate the tumor. A number of studies support this theory (reviewed in (1). Our studies show that immune cells infiltrating the primary tumor included $CD4^{+}$T cells, macrophages and NK cells, suggesting their involvement in anti-tumor activity in this system. This is a wider repertoire of immune cells compared to when either GDEPT is used alone. The distant bystander effects from CD- and UPRT-GDEPTs are mediated by the immune system

(14,15,34). Thus UPRT expressing murine colon carcinoma cells in syngeneic immunocompetent mice led to tumor regression when treated with 5FU compared with wild type tumor cells (14). Treated mice rejected the wild type- but not irrelevant syngeneic tumor cells. This distant bystander effect was less efficient in nude mice suggesting that αβ T cells were involved. In comparison, in a rat model that mimics liver metastases of colon carcinoma, CD GDEPT led to regression of CD positive tumors (25,34) and resistance in treated rats to wild type challenge. Immunodepletion studies showed that NK cells were involved. This would suggest that NK and αβ T cells are implicated in CD and UPRT GDEPT effects, respectively. Hence, when the two systems are combined the immune system may be augmented by the inclusion of a larger repertoire of anti-tumor immune cells. Our preliminary analyses support this, but immunodepletion studies are needed to ascertain the mechanisms involved.

Hemorrhagic necrosis was a remarkable feature of CDUPRT/5FC treated RM1-GFP/CDUPRT tumors (Fig 2C,Table 1) suggesting that endothelial cells in the tumor vasculature were susceptible to GDEPT. This disruption could be mediated by the cytokines released due to the stimulation of the immune system (33) or due to the toxicity of 5FU released in the tumor microenvironment (35). G1-arrest in endothelial cells was observed when treated with 5FU (39). In our study, haemorrhagic necrosis increased in a dose dependant manner with GDEPT treatment (Table 2), strongly suggesting that disruption of tumor vasculature may have contributed further to the tumor regression in CDUPRT treated mice. The elucidation of the mechanism of this disruption however, was beyond the scope of this study.

Killing of tumor cells by anticancer therapies such as chemo-, radiation-, immuno- or suicide gene therapy is predominantly mediated by triggering apoptosis. Our data indicated that apoptosis was involved in the death of RM1 tumor cells expressing CDUPRT (Fig. 5) and again the dose dependence of this effect suggested that it was CDUPRT GDEPT-specific. This accords with other studies showing the involvement of apoptosis in cell killing mediated by various GDEPTs such as HSV/TK, PNP and CD (23,36-38).

Although the CDUPRT-GDEPT has not yet been trialled in humans, the safety of the CD-GDEPT has been well reported in PC patients (39). In this preclinical study, there was no apparent toxicity to other organs indicating that CDUPRT-GDEPT has excellent safety features against normal host tissues. There is increasing emphasis on use of gene therapy in concert with other strategies, including strategies that enhance anti-tumor immunity. Our data indicate that CDUPRT/5FC GDEPT significantly suppressed the growth of RM1 cells producing both a local and a distant bystander effect by mechanisms of killing that involve necrosis and apoptosis possibly mediated by the immune responses. This proof of principle study will form the basis of the future studies assessing the impact of combination of CDUPRT GDEPT with immunotherapy.

## 1. MATERIALS AND METHODS

### Cell lines and mice

The RM1 (16) and the transformed derivatives (RM1-CDUPRT, RM1-GFP and RM1-LacZ) were cultured in Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen, CA, USA) containing 10% fetal calf serum (Invitrogen, CA, USA) with hygromycin (hygro) (Invitrogen, CA, USA) at a concentration of 800μg/ml for the later. Male C57BL/6 (6-8wk) mice were bought from Labortory Animal services, Perth, AU.

### Transfection of RM1 cells

RM1 cells were transfected with pVITRO2-GFP/CDUPRT, pVITRO2-GFP/LacZ (Invivogen, CA, USA) or pVITRO2-GFP (InvivoGen, CA, USA) to generate stable transfectants (RM1-CDUPRT, RM1-LacZ and RM1-GFP). The pVITRO2-GFP/CDUPRT was constructed by

excision of CDUPRT (CodA::upp) gene from pORF-codA::upp (InvivoGen, CA, USA) using *NcoI* and *NheI* restriction enzymes followed by its ligation into complementary sites in the pVITRO2-GFP/LacZ (InvivoGen, CA, USA). The construct was authenticated by restriction enzyme digestion using *NcoI* and *NheI*. For transfections, cells were transfected with complexes formed by combining 15µl Lipofectamine 2000 (Invitrogen, CA, USA) and 5µg plasmid DNA according to manufacturer's instructions. Stable clones were maintained under hygro B selection (800µg/mL). GFP expression was used to sort the cells (FACscan sorter, BD, USA) for high levels of GFP expression and to eliminate drug-resistant, non-expressing clones.

**Optimization 5FC dose *in vivo***

Mice injected ip with 150, 300 and 500 mg/kg/mouse/day of 5FC (InvivoGen, CA, USA) or saline for 13 days were monitored daily for general behaviour and condition and their body weights were measured every second day. Toxic effects were monitored by assessment of liver and renal function via evaluation of the levels of urea, creatinine, alkaline phosphatase (ALP), alanine amino-transferase (ALT) and aspartate amino-transferase (AST) in the serum samples by the South East Area Laboratory Services (Prince of Wales Hospital, AU) using standard techniques.

**Assessment of CDUPRT expression *in vitro* and *in vivo***

To assess the functionality of CDUPRT in RM1-CDUPRT cells/tumours, an HPLC based assay was developed measuring the catabolism of the prodrug 5FC to 5FU. Homogenates of RM1-CDUPRT or –LacZ cells sc/intraprostatic (Iprost) RM1-CDUPRT or –LacZ tumors (Liquid $N_2$) were generated and lysis was completed by 3 cycles of freeze thawing. Cell debris was removed by centrifugation (15,000g, 10 min) followed by determination of the protein content of the supernatants (BCA protein estimation kit, Pierce, IL, USA). 100µL of the supernatant was then incubated with 900 µL of 0.5 mM 5FC at 37°C. At 24 h, the samples were stored at –20°C, after a 10 min incubation at 85°C. Reversed phase liquid chromatography employing a C18 column under isocratic conditions (0.05% Trifluoroacetic acid in $H_2O$) at a flow rate of 0.7 mL/min was used to analyse samples (10 µL). Absorbance was measured at 275 nm. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU.

**GDEPT *in vivo***

Iprost injections were performed with $5x10^3$ RM1-CDUPRT (test) or RM1-LacZ (control) cells in the subcapsular region of the prostate surgically after opening the abdomen in C57BL/6 mice as previously described (23). Day 4 onwards, 5FC or saline was administered ip at 500 mg/ kg/day for 14 days. Mouse weights were recorded twice/week. At necroscopy (day 18), the prostate tumor volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$ where $d_1$ and $d_2$ are diameters at right angles (40). The tumor and other organs (kidney, lungs, liver, heart, spleen) were fresh frozen or paraffin-embedded for subsequent histological and immunohistochemical studies. Mouse serum was stored at –80°C until analysis.

**Local bystander effect on RM1 growth**

*In vitro*: Conditioned media from RM1-CDUPRT cells +/- 5FC (at 1mM) or RM1-LacZ cells+5FC were harvested 48 h after the addition of 5FC. These mixed with an equal proportion of fresh medium were then incubated with RM1 parental cells ($5x10^3$ cells/well in a 96 well plate). Cell viability was determined at 72 h using the WST1 proliferation assay (Roche, Sydney, AU) according to the supplier's instructions.

*In vivo:* RM1-CDUPRT cells and RM1-GFP cells were mixed in different proportions and implanted Iprost in mice followed by 5FC treatment daily for 14 days. As data from our earlier experiments showed the GDEPT to be specific to RM1-CDUPRT/5FC group, to minimize the mouse usage, the saline controls included earlier were not performed. On day 19, tumors and other organs were processed as described above.

**Effects of RM1-CDUPRT plus 5FC on growth of pseudometastases in the lungs**

Mice were injected Iprost with $5x10^3$ RM1-CDUPRT or RM1-GFP cells. On day 4, mice received $2.5x10^5$ RM1 cells iv and 5FC treatment daily ip for 15 days. On day 19, their lungs were fixed in Bouin's fixative and the RM1 colonies were counted as previously described (23).

**Immunohistochemical analysis of orthotopically implanted prostate tumors**

*For detection of Immune infiltration:* Snap frozen tissues were embedded in Optimal Cutting Temperature compound (Tissue Tek), sectioned (5 μm) and acetone fixed. To block endogenous peroxidase/biotin and non-specific monoclonal antibody binding, sections were incubated sequentially with 1.5% $H_2O_2$ (5 mins), avidin block (10 mins), biotin block (10 mins), 3% bovine serum albumin in PBS (5 mins). Sections were then stained using rat α mouse -CD4 (BD-PharMingen, 1:100); -CD8a (BD-PharMingen, 1:200); -F4/80 (BD-PharMingen,1:800), -CD31 1:300, BD-Pharmingen) and rabbit α mouse AsialoGM1 (Dako,1:400) as relevant by incubating for 45 minutes at room temperature followed by incubation with secondary antibodies, α -rat (1:200) and α-rabbit (1:200) and the ABC complex for 15 minutes. The standard ABC detection system, the diaminobenzidine (DAB) as the chromagen and Harris hematoxylin as counterstain were used.

*For detection of Apoptosis*: Tissues fixed in 10% neutral buffered formalin (Amber Scientific) were paraffin-embedded (Tissue Tek-VIP (Sakura). Dewaxed paraffin sections (Histochoice, Ambresco) were rehydrated through graded series of ethanol and finally in PBS before blocking. Apoptotic cells were detected using the Tunel assay kit (Roche) as described (41) after antigen retrieval.

Scoring of positive stained cells in the immunostained sections was performed by light microscopy. After initial scanning under x100 magnification, positive stained cells in ten fields under x400 (0.15 $mm^2$) magnification were counted and the mean number/high power field (HPF ± SEM) was determined. Because of the high number of positive apoptotic cells, a higher magnification (x620) was used.

**Data analysis**

A one-way analysis of variance (ANOVA) was performed (GraphPad PRISM V4) if the data in multiple groups were normally distributed. A Turkey's post-test was performed if the ANOVA indicated a significant difference ($p<0.05$) between treatments.

**ACKNOWLEDGEMENTS**

This work was supported by DOD Prostate Cancer grant, DAMD-17-1-02-0107, and by the DOD fellowship, DAMD-17- 01-1-0083.

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Table 1: Effects of CDUPRT-GDEPT on prostate histology.

| Treatment<br>5 x $10^3$ RM1-GFP/CDUPRT (Iprost) | Viability | Necrosis | Haemorrhagic[a] Necrosis |
|---|---|---|---|
| + saline ip 15 days (n=6) | >80% | <10% | <10% |
| + 5FC (500 mg/kg/day) ip daily for 15 days (n=4) | <10% | >30% | >60% |
| + 5FC (500 mg/kg/day) ip daily for 15 days (n=6) | No | No growth | No growth |

[a] Haemorrhagic necrosis was defined by necrotic cell death observed in areas with vascular damage, primarily observed in tumours treated with CDUPRT-GDEPT.

**Table 2.** Immunohistochemical analysis of infiltrating immune cells, endothelial cells (vascularity) and apoptotic tumor cells in RM1-GFP/CDURPT+5FC and control tumors.

| Treatment | Number of Infiltrating immune cells | | | | | | | | Vasculature (endothelial cells) | | Apoptotic tumor cells | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $CD4^+$ | | $CD8a^+$ | | $F4/80^+$ | | $AsialoGM1^+$ | | CD31 | | Tunel assay | |
| | Mean[a] | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| **(A) RM1-GFP+5FC (Control)** | 4.55 | 0.39 | 1.26 | 0.40 | 10.85 | 1.90 | 13.05 | 1.10 | 18.03 | 2.89 | 47.03 | 3.74 |
| **(B) 10%RM1-GFP/CDURPT mixed with 90%RM1-GFP +5FC** | 9.43 | 3.36 | 4.25 | 1.27 | 20.53 | 0.76 | 14.93 | 1.82 | 13.82 | 2.09 | 65.30 | 0.62 |
| **(C) 20%RM1-GFP/CDURPT mixed with 80% RM1-GFP +5FC** | 9.40 | 3.46 | 1.73 | 0.90 | 16.23 | 2.38 | 16.20 | 2.76 | 8.33 | 0.13 | 72.97 | 2.17 |
| **(D) RM1-GFP/CDURPT (100%) +5FC** | 32.9 | 8.9 | 1.70 | 1.70 | 22.00 | 4.71 | 20.27 | 3.94 | 5.67 | 0.35 | 84.25 | 0.75 |
| **One way ANOVA P value** | 0.003 | | 0.19 | | 0.002 | | 0.04 | | 0.0061 | | 0.0001 | |

[a] Mean number of positively stained cells/high power field (HPF ± SEM) (x400) (0.15 mm square) from a count of 10 fields. SEM: Standard error of the mean.

**Figure Legends**

Figure 1: Analysis of toxicity of 5FC in C57BLl/6 mice. Mice (4 mice /group) were injected ip with 100, 150 and 500 mg/kg/day of 5-FC or saline for 13 days. At necroscopy, heart, liver, lung and spleen were harvested and analysed by H&E staining for toxic effects of the drug. (A)The four panels show different organs from mice treated at the highest dose, 500mg/kg/day. Insets in each panel represent the corresponding organ from control mice treated with saline. There was no detectable toxicity even at 500mg/kg/day. (B) serum analysis for biochemical markers of kidney and liver function (ALP: Alkaline Phosphatase, ALT: Alanine Amino Transferase and AST: Aspartate Amino Transferase is shown. The reference represents the values for a normal mouse.

Figure 2: *In vivo* evaluation of therapeutic effects of CDUPRT-GDEPT using RM1-GFP/CDUPRT cells +5FC

(A) 5x103 cells were implanted orthotopically in the prostate of C57BL/6 mice. 4 days post-implantation the prodrug 5FC or saline were administered ip at 500mg/kg/mouse/day for 13 days. At necroscopy (day 17), prostate tumor volume was measured. Prostate volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where $d_1$ and $d_2$ are diameters at right angles.

(B) H&E staining of paraffin-embedded orthotopic RM1-CDUPRT prostate tumor sections show that treatment with saline resulted in highly vascularized viable tumor ( x40), (C) Treatment with 5FC resulted in extensive necrosis (black arrow) and haemorrhagic necrosis (red arrow) (C x40), with some loss of prostate tissue architecture (insert, x10).

Figure 3: Evaluation of "local bystander effect" of CDUPRT *in vitro* and *in vivo.*

(A) *In vitro*: RM1-GFP/CDUPRT cells were grown in presence or absence of 5FC for 48 h, then the supernatants were collected. Supernatants (conditioned media, CM) from RM1-GFP/LacZ cells grown in the presence of 5FC, served as controls. Parental RM1 cells were then treated with these CMs at 50% concentration. The bystander effect was demonstrated by cell killing of parental RM1 cells using theCM from RM1CDUPRT cells using WST1 viability assay (see methods).

(B) *In vivo*: Local bystander cell killing effects of the CDUPRT suicide gene is demonstrated. RM1-GFP/CDUPRT cells were mixed with RM1-GFP cells in different proportions and $5x10^3$ total cells were implanted in the prostate of C57BL/6 mice. The mice were injected intraperitoneally with the prodrug, 5-fluorocytosine (5FC) at 500 mg/kg/day, from day 4 onwards

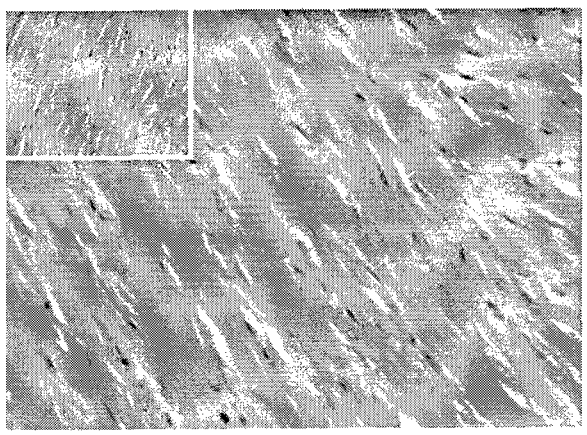
daily for 15 days. Mice were sacrificed on day 19, and their prostate volumes determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where $d_1$ and $d_2$ are diameters at right angles.

Figure 4: Assessment of apoptosis and vascular integrity in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40): (A) Tunel positive cells in test tumor vs Inset, Tunel stain control tumor; (B) Decrease in CD31 positive cells in RM1-GFP/CDUPRT + 5FC tumor vs Inset, CD31 positive cells in control tumor.

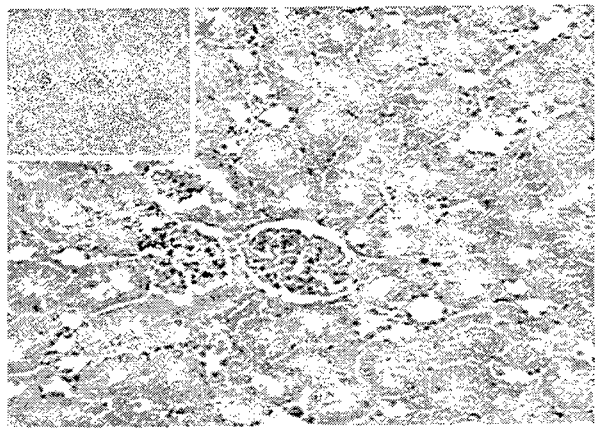
Figure 5: Distant bystander effect of CDUPRT suicide gene *in vivo*. RM1-GFP/CDUPRT cells ($5x10^3$) were implanted in the prostate of C57BL/6 mice. Four days later, the mice were injected intravenously with the parental RM1 cells at the dose of $2.5x10^5$ cells/mouse. The mice were injected with the prodrug 5FC from day 4 onwards daily for 15 days. At necroscopy, the lungs were harvested, stored in Bouin's reagent and colony counts were performed. (A) Scatter graph showing the number of lung colonies in different treatment groups (B) Photographs of lungs demonstrating the distant bystander effect of CDUPRT GDEPT on pseudometastases.

Figure 6: Evaluation of immune cell infiltration after treatment in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40) (A) Cluster of CD4 positive T cells (brown colour) in test tumor; Inset few CD4 positive T cells in control tumor.; (B) Cluster of F4/80 positive macrophages in test tumor. vs Inset: control tumor; (C) Asialo-GM1 positive immune cells clustered at the tumor periphery in test tumor vs Inset, Control tumor.

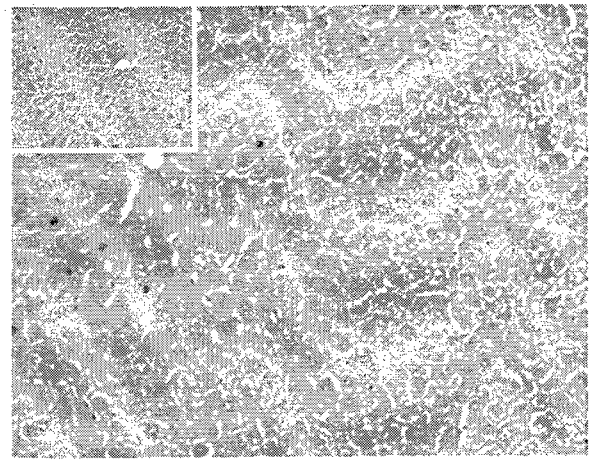
A



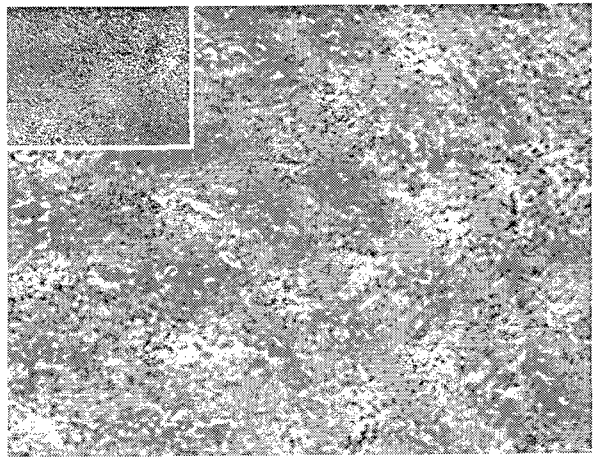
Heart



Kidney



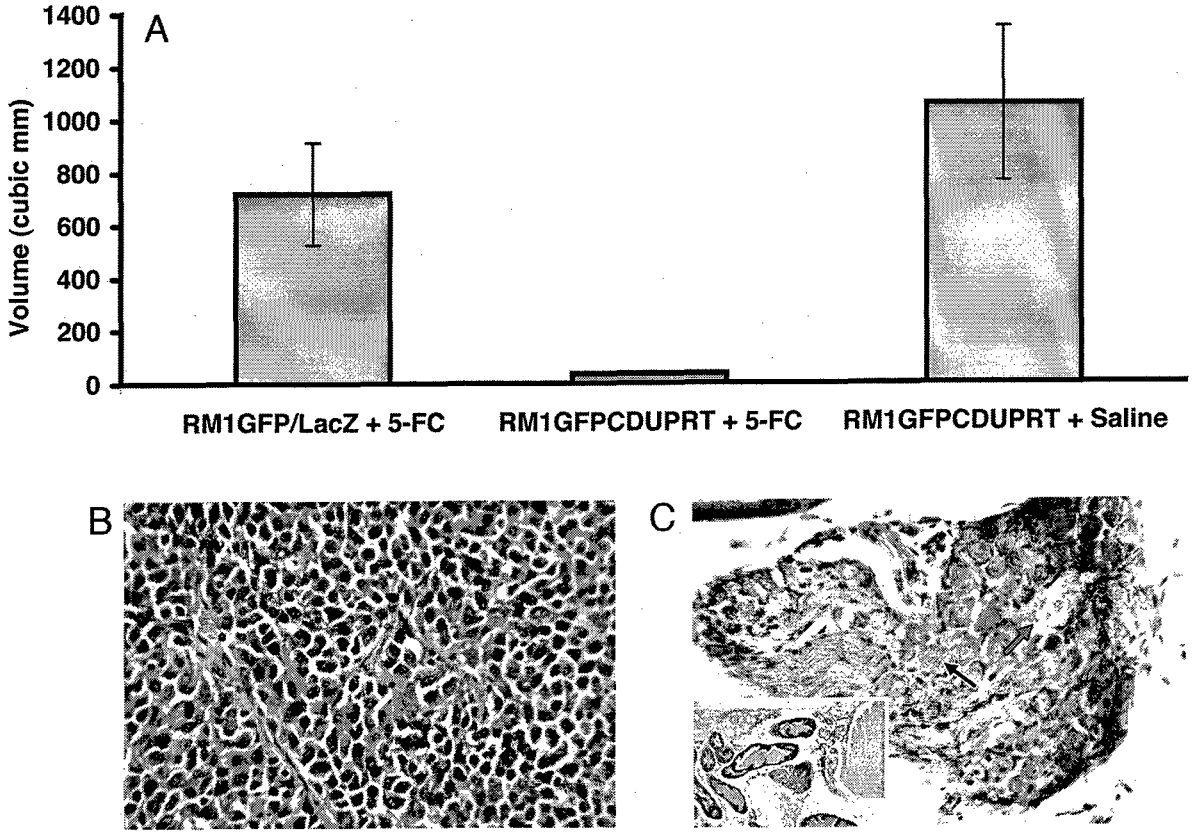
Liver

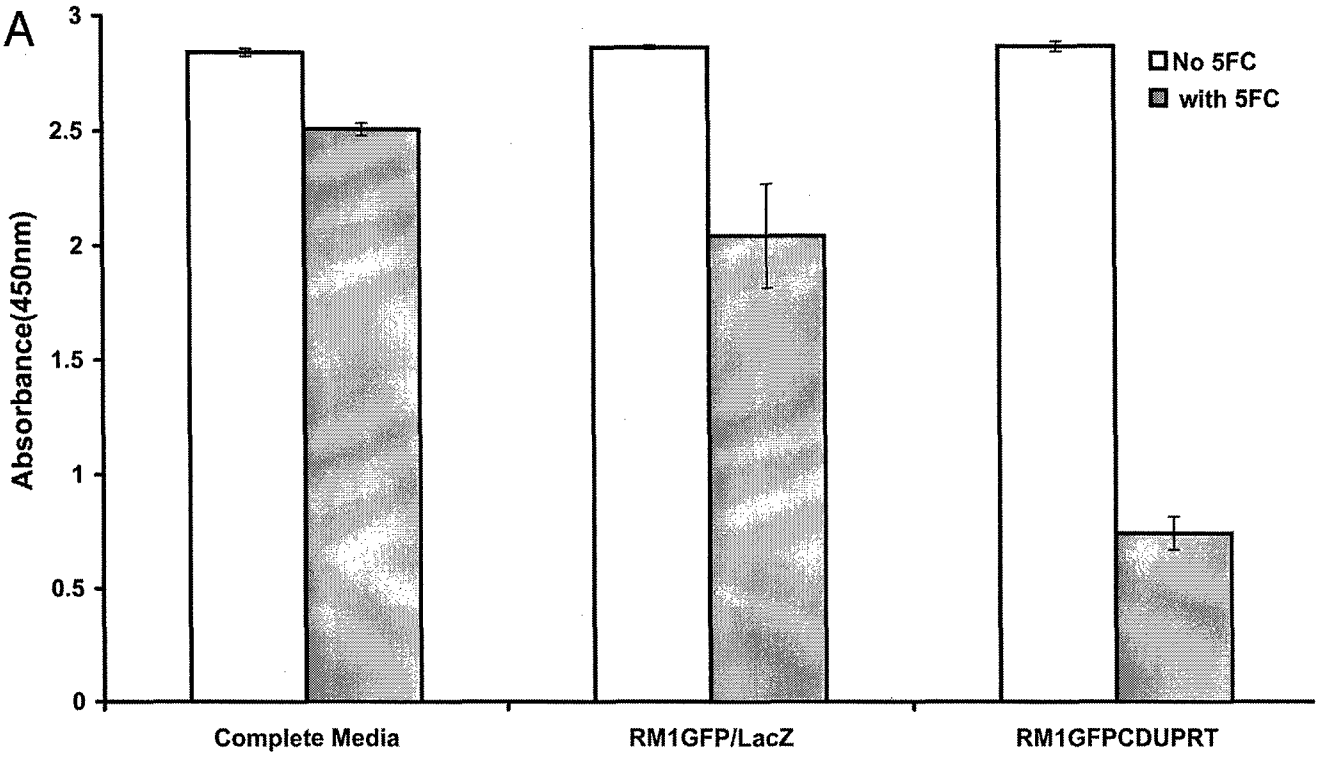


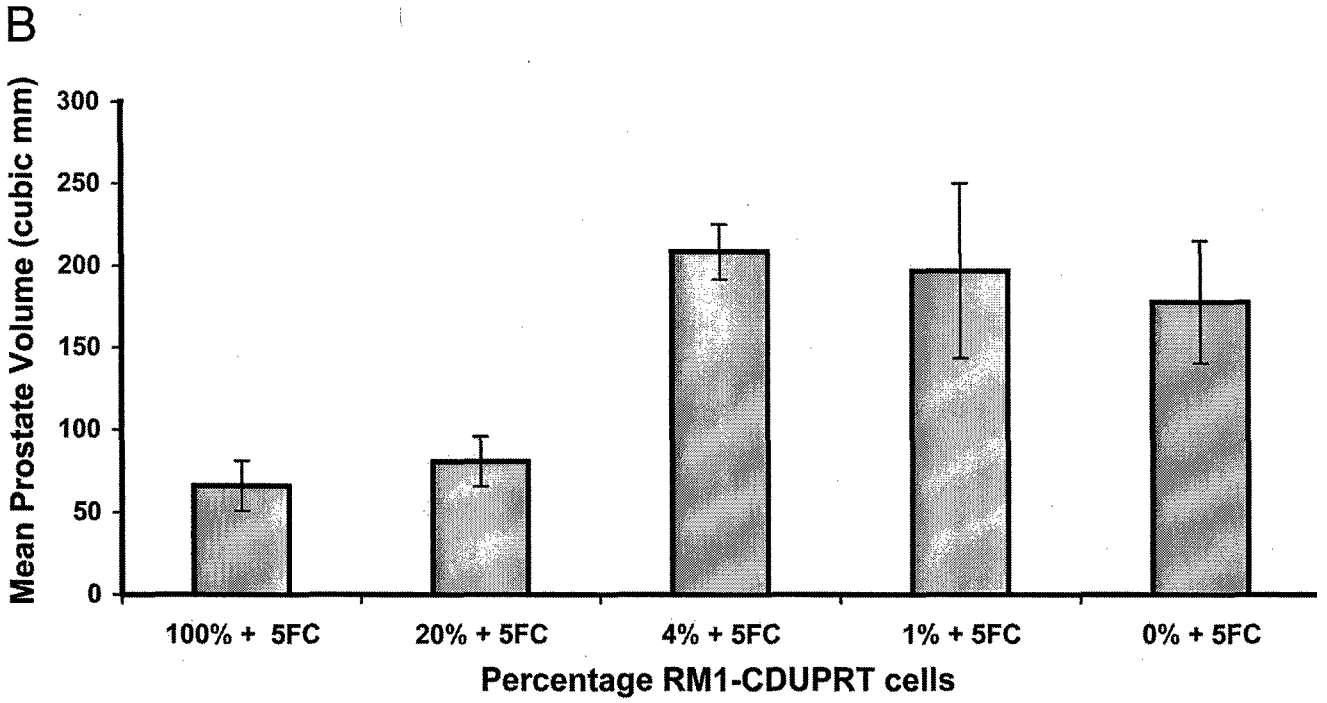
Spleen

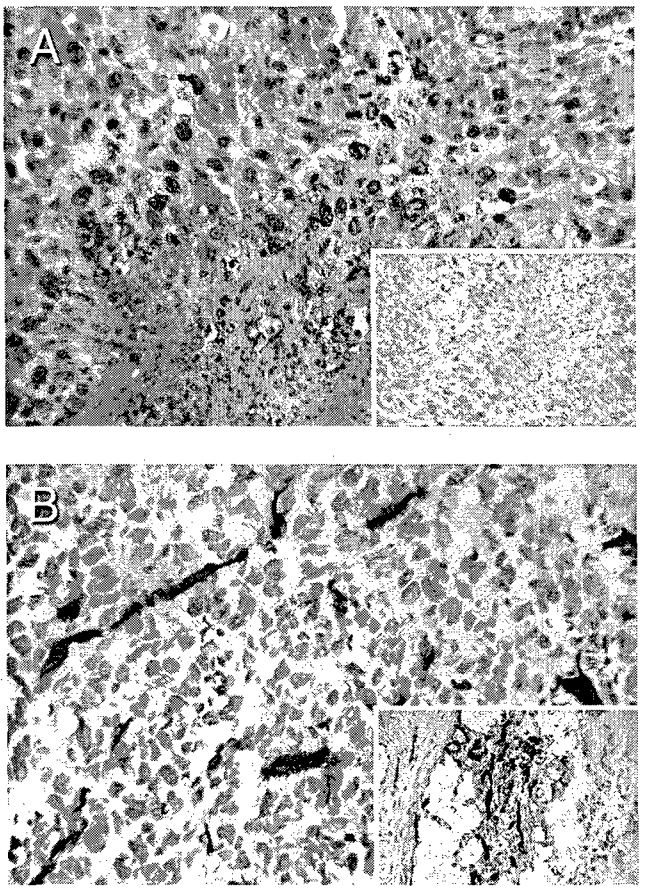
B

| Treatment | Urea (mmol/L) | Creatinine (umol/L) | ALP (U/L) | ALT (U/L) | AST (U/L) |
|---|---|---|---|---|---|
| **Reference** | **1.4-5.5** | **18-80** | **35-96** | **17-77** | **54-298** |
| Control | 6.7 ± 0.3 | 29.3 ± 3.3 | 54 ± 1.7 | 32.5 ± 5.7 | 155 ± 25 |
| 500 mg/kg/d | 6.8 ± 0.7 | 25.3 ± 1.8 | 55.7 ± 4.9 | 19 ± 1.5 | 57.3 ± 11.9 |

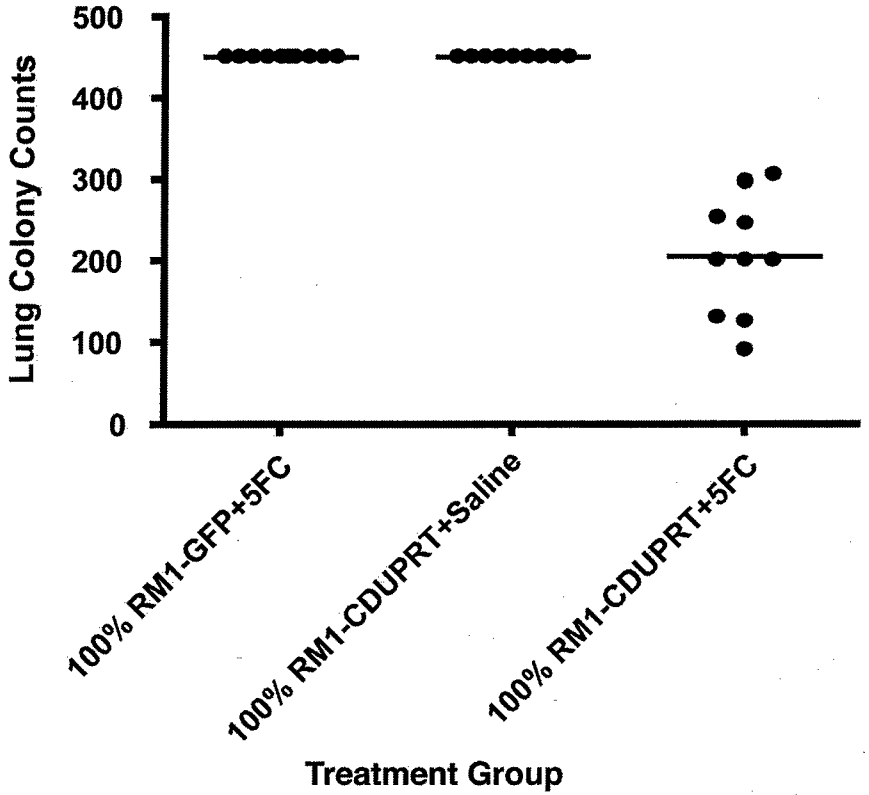

A
1400
1200
1000
800
600
400
200
0
Volume (cubic mm)
RM1GFP/LacZ + 5-FC
RM1GFPCDUPRT + 5-FC
RM1GFPCDUPRT + Saline
B
C


A
Absorbance(450nm)
3
2.5
2
1.5
1
0.5
0
No 5FC
with 5FC
Complete Media
RM1GFP/LacZ
RM1GFPCDUPRT

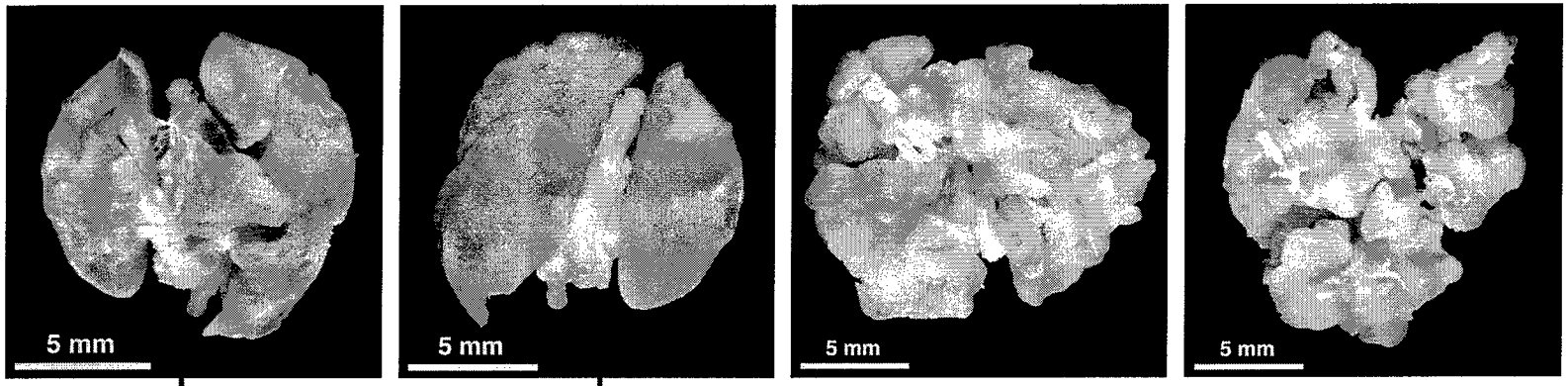

B
Mean Prostate Volume (cubic mm)
300
250
200
150
100
50
0
100% + 5FC
20% + 5FC
4% + 5FC
1% + 5FC
0% + 5FC
Percentage RM1-CDUPRT cells

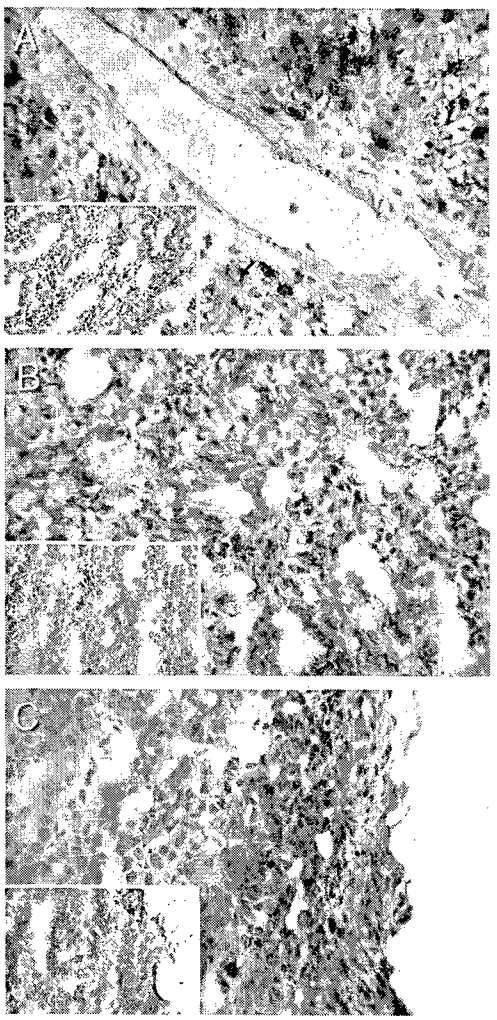

A
B

A


500
400
300
200
100
0
Lung Colony Counts
100% RM1-GFP+5FC
100% RM1-CDUPRT+Saline
100% RM1-CDUPRT+5FC
Treatment Group

B


5 mm
5 mm
5 mm
5 mm
RM1CDUPRT/5FC
RM1CDUPRT/saline
RM1GFP/5FC


A
B
C

APPENDIX 2

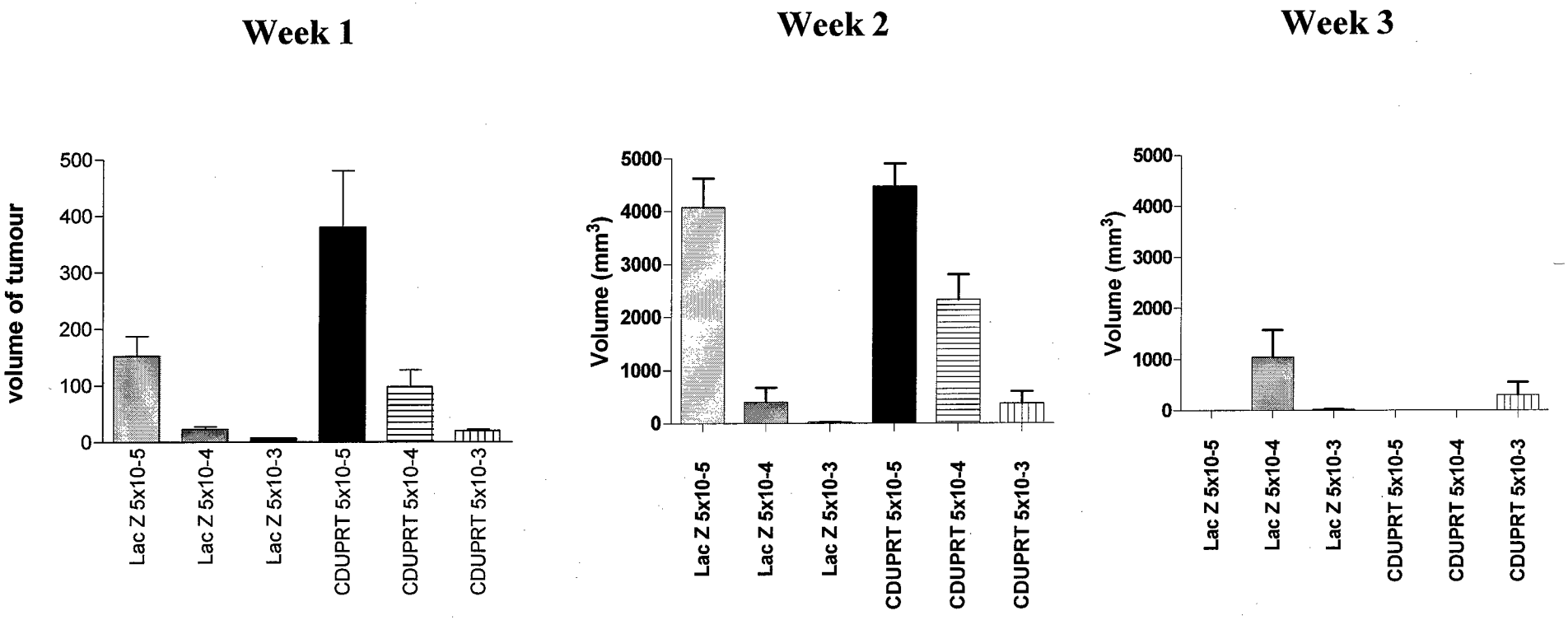



Figure 1: Growth of RM1CDUPRT and RM1LacZ tumors in the prostate of C57BL/6 mice. Mice were injected intraprostatically (Iprost) with different doses of RM1CDUPRT or RM1LacZ cells and the tumor growth was measured at weeks 1, 2 and 3. By week 3, mice with large tumors had to be culled (RM1LacZ, $10^5$ cells and RM1CDUPRT $10^4$ and $10^5$ cells)

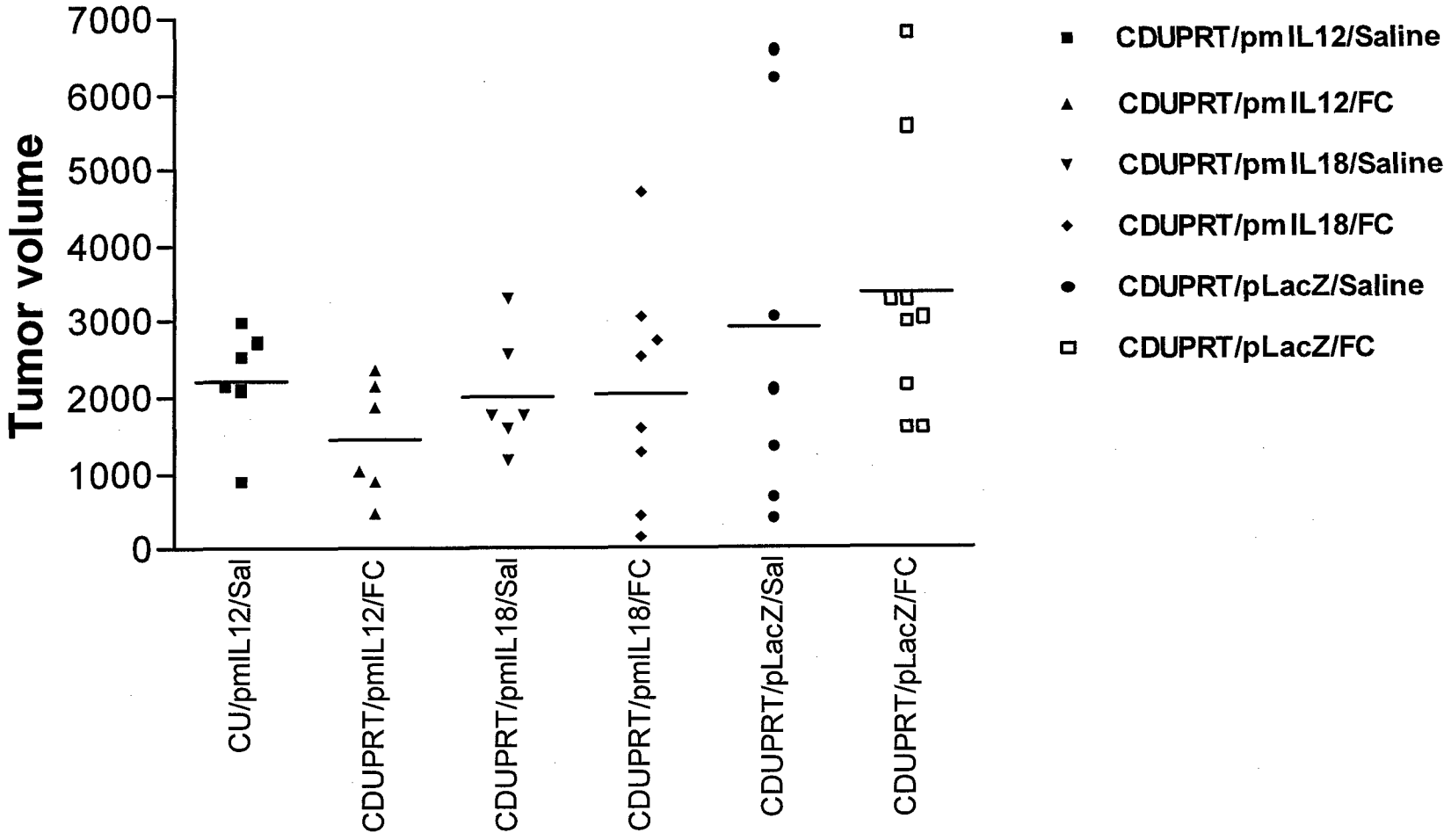



Figure 2: **Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 or pVITRO2.mIL18 on growth of intraprostatic (Iprost) RM1 tumors in C57BL/6 mice. Mice implanted with RM1CDUPRT tumors were injected intratumorally (i.t.)with pVITRO2.mIL12 or pVITRO2.mIL18 or pVITRO2.LacZ followed by intraperitoneal (ip)injections of 5FC or saline everyday until necroscopy. A comparison of tumor volumes between different groups is shown**

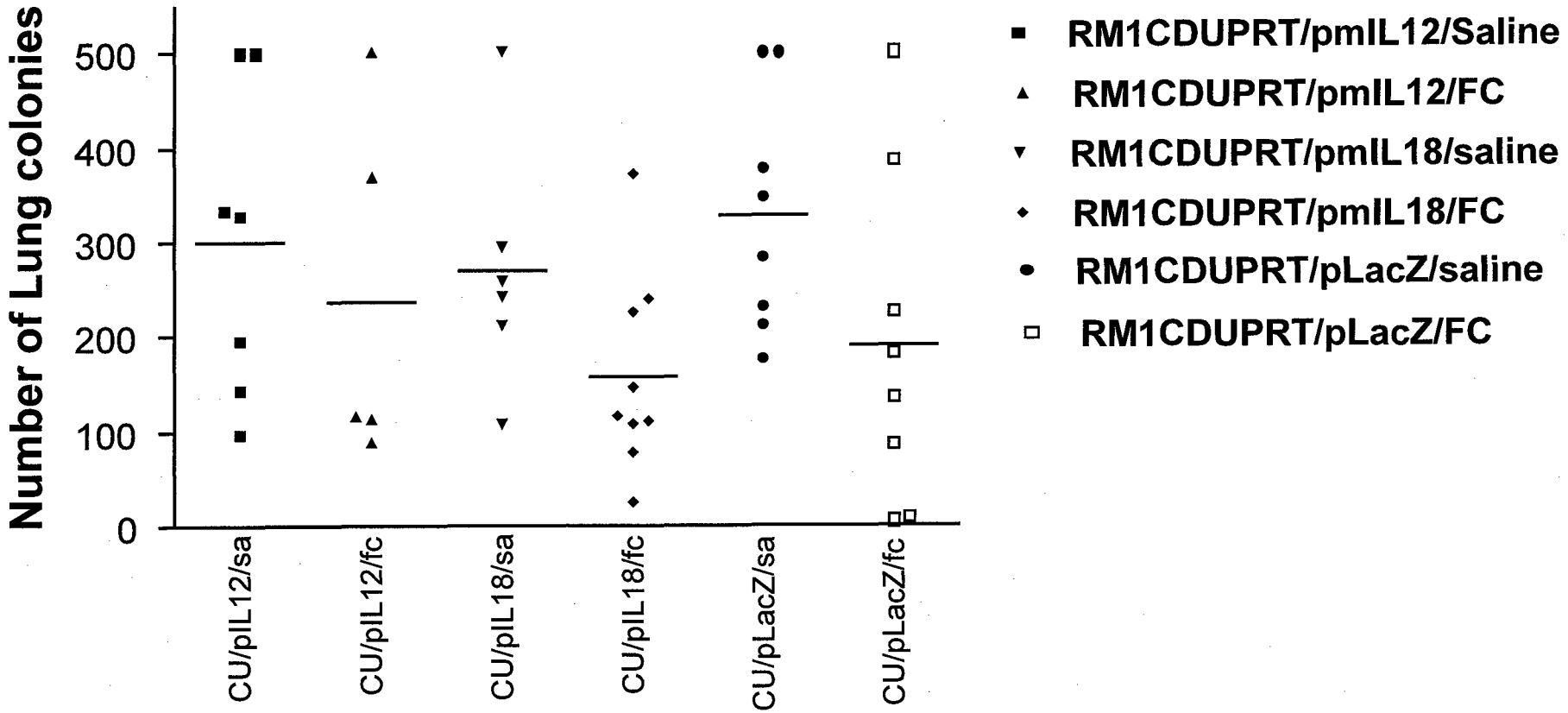



Figure 3: **Distant Bystander effect: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 or pVITRO2.mIL18 in treatment of RM1 psuedometastases in lungs of C57BL/6 mice. Mice implanted with RM1CDUPRT tumors were injected i.t. with pVITRO2.mIL12 and pVITRO2.mIL18 or pVITRO2.LacZ . On day 6. mice were injected intravenously (iv) with parental RM1 cells to establish psuedometastases in lungs and 5FC or saline were given ip daily until necroscopy. At necroscopy, lungs were fixed in Bouin's reagent and colony count was done under the dissecting microscope. Lung colony count of different treatment groups is shown.**

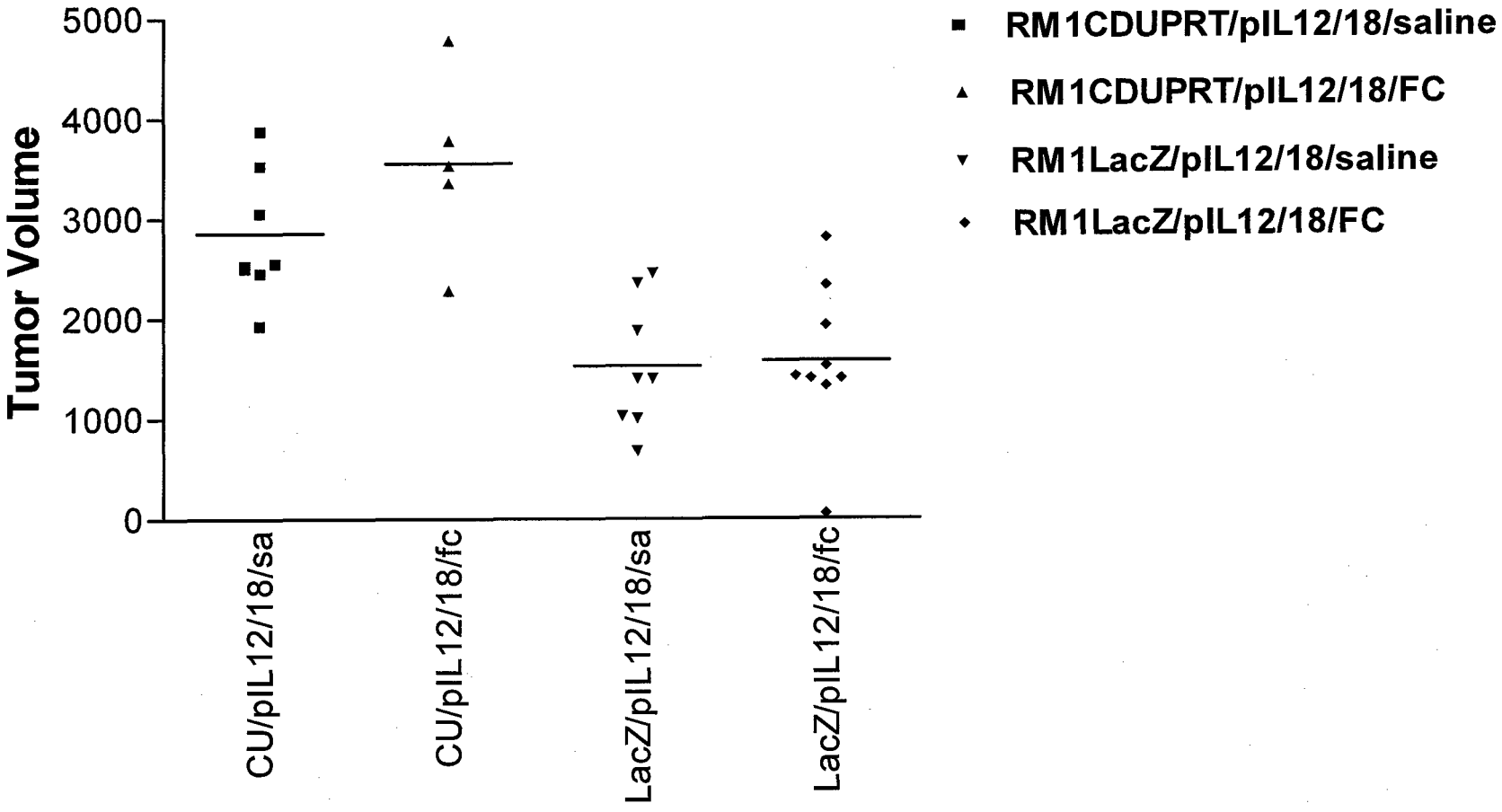



**Figure 4: Effects of CDUPRT GDEPT in combination with pVITRO2.mIL12 and pVITRO2.mIL18 on growth of intraprostatic RM1 tumors in C57BL/6 mice.** **Mice implanted with RM1CDUPRT tumors were injected i.t. with pVITRO2.mIL12 and pVITRO2.mIL18 or pVITRO2.LacZ followed by 5FC or saline treatment daily until necroscopy.**

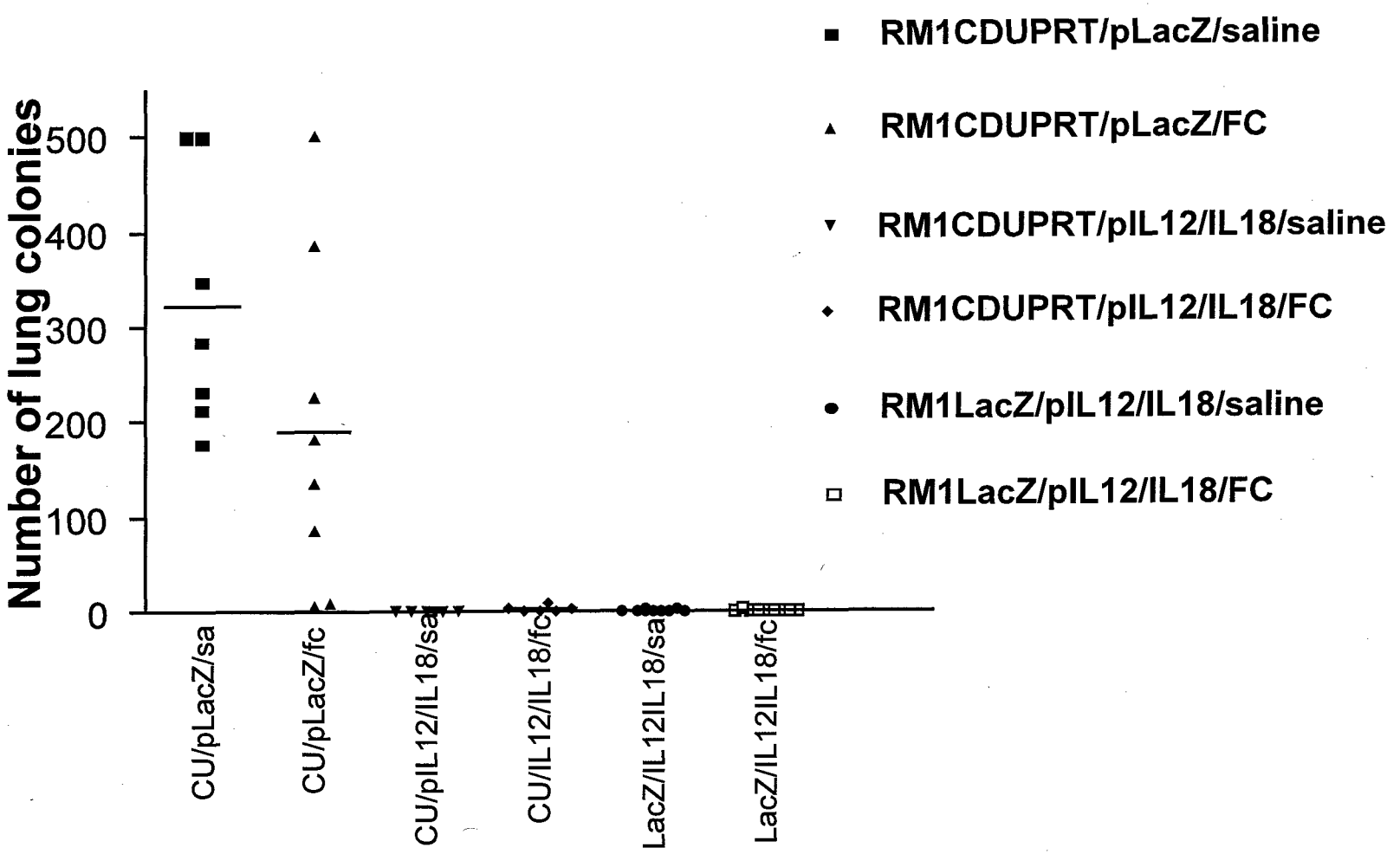



Figure5: **Distant Bystander effect of the trimodal therapy: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 and pVITRO2.mIL18 on RM1 pseudometastases in C57BL/6 mice. Mice implanted with RM1CDUPRT tumors were injected i.t. with pVITRO2.mIL12 and pVITRO2.mIL18 or with pVITRO2.LacZ . On day 6. mice were injected iv with parental RM1 cells to establish pseudometastases in lungs and 5FC or saline were given ip daily until necroscopy. Lungs were then fixed in Bouin's reagent and colony counts were done. Lung colony counts in different treatment groups are shown. RM1LacZ cells and pVITRO2.LacZ plasmid were used as controls.**

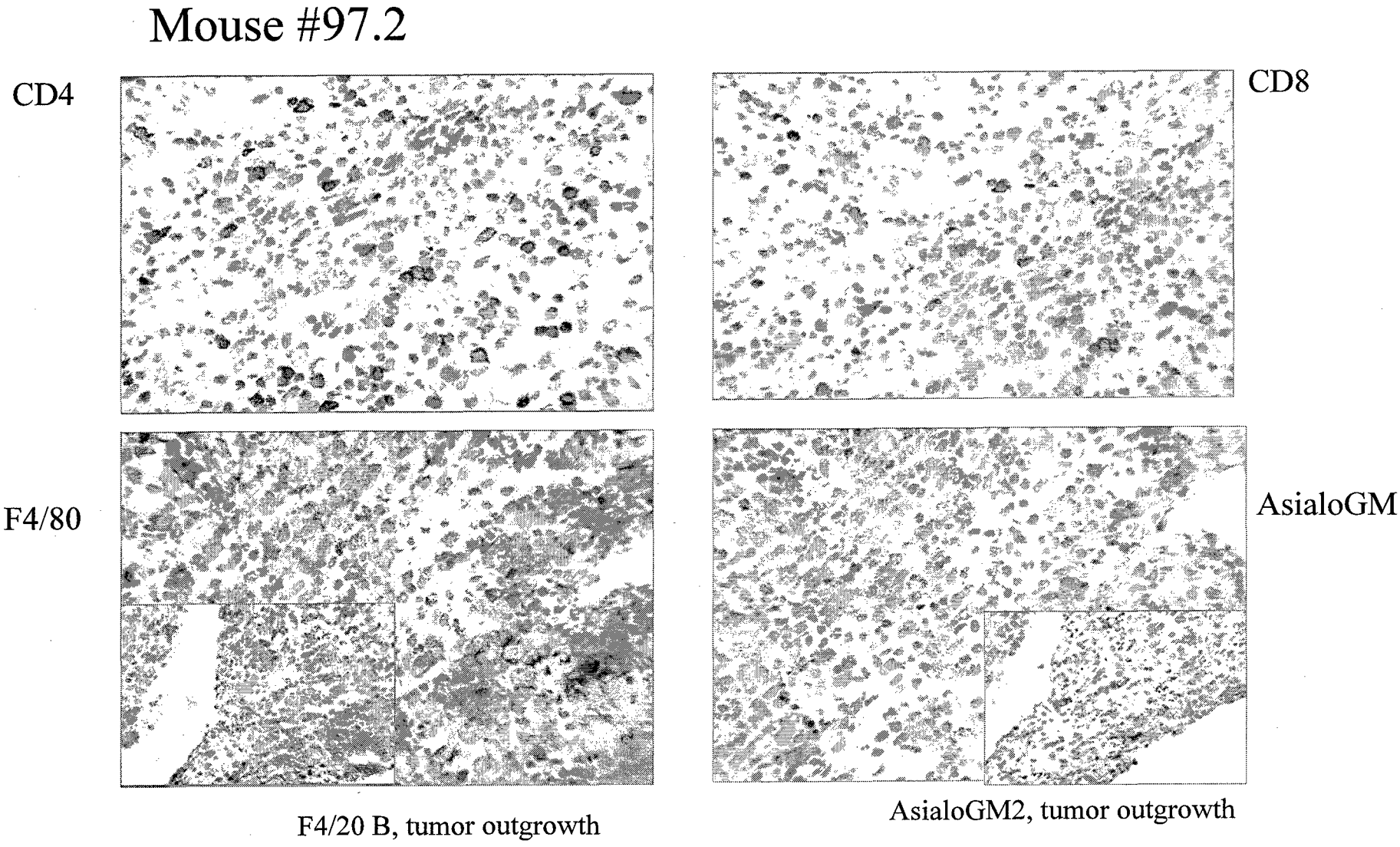
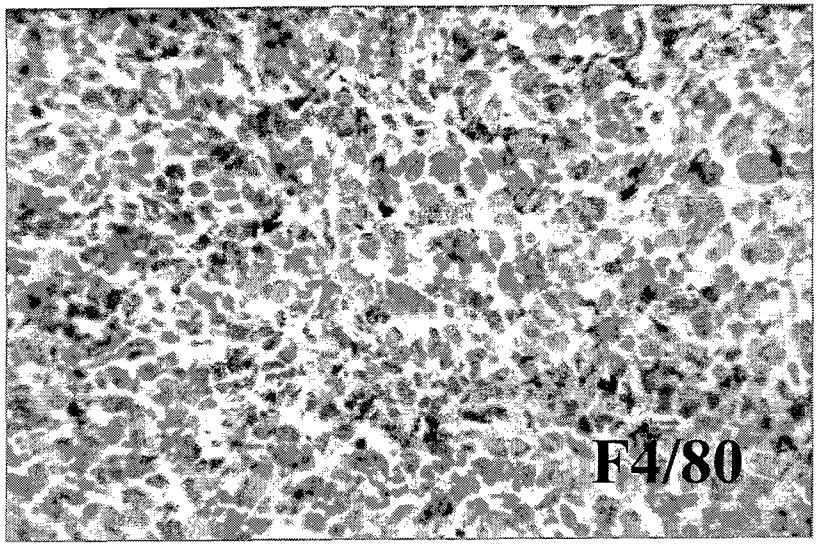
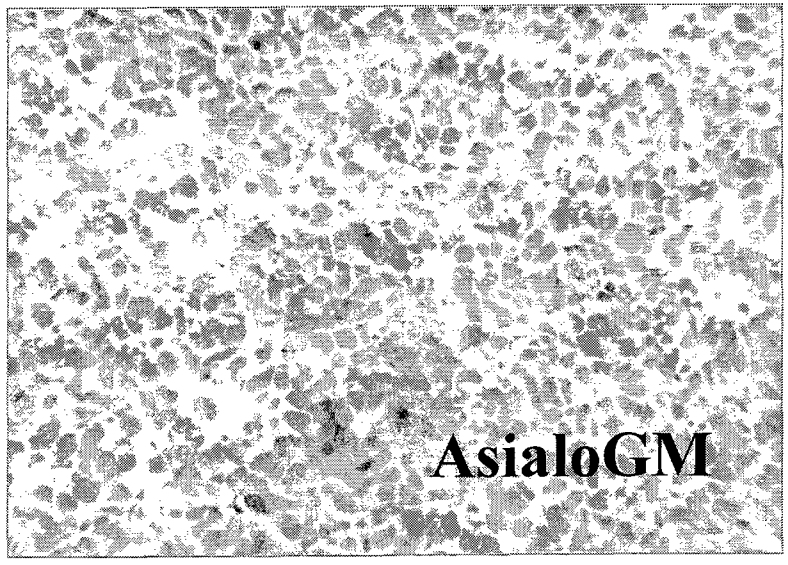



Figure 6: Infiltration of RM1LacZ prostates treated with pVITRO2.mIL12 plus pVITRO2.mIL18 (i.t.) and given 5FC daily ip until euthanasia. CD4+ and CD8+ T cells, F4/80+ macrophages and AsialoGM+ cells were present. An additional tumour outgrowth was rich in macrophages, but not NK cells (inserts)

Mouse #103.2



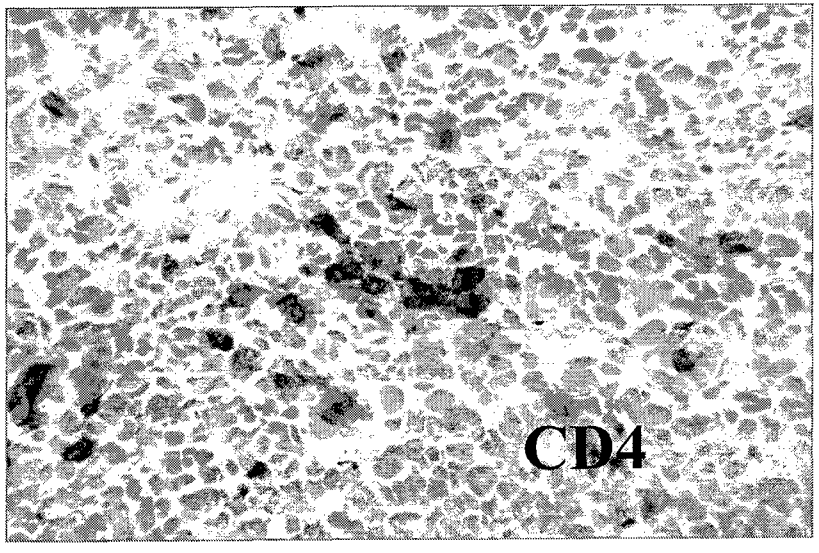
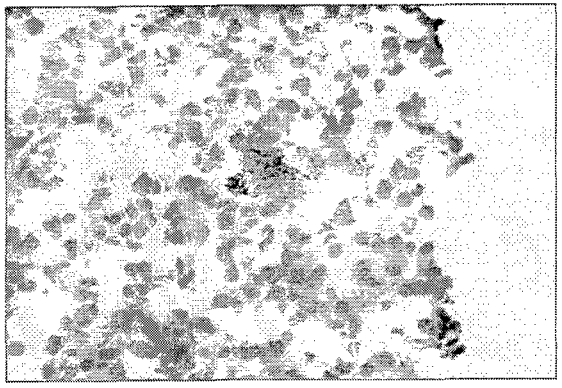
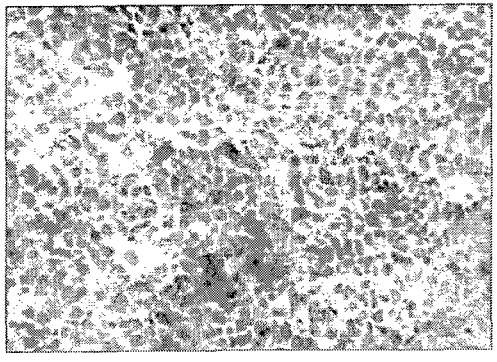




Figure 7: RM1LacZ iprost tumors treated with IL12.IL18 (i.t.) then saline ip until euthanasia were infiltrated with macrophages (F4/80) NK (AsialoGM) and CD4 T cells.

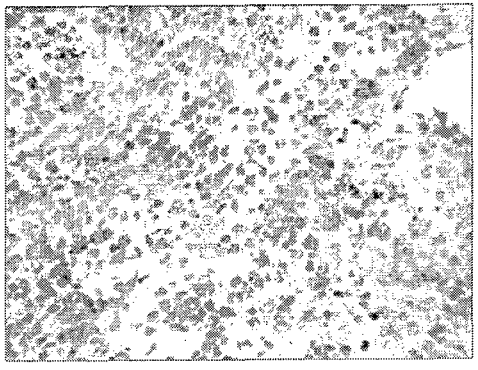
## Asialo GM



**RM1CDUPRT/IL12/IL18/5FC**

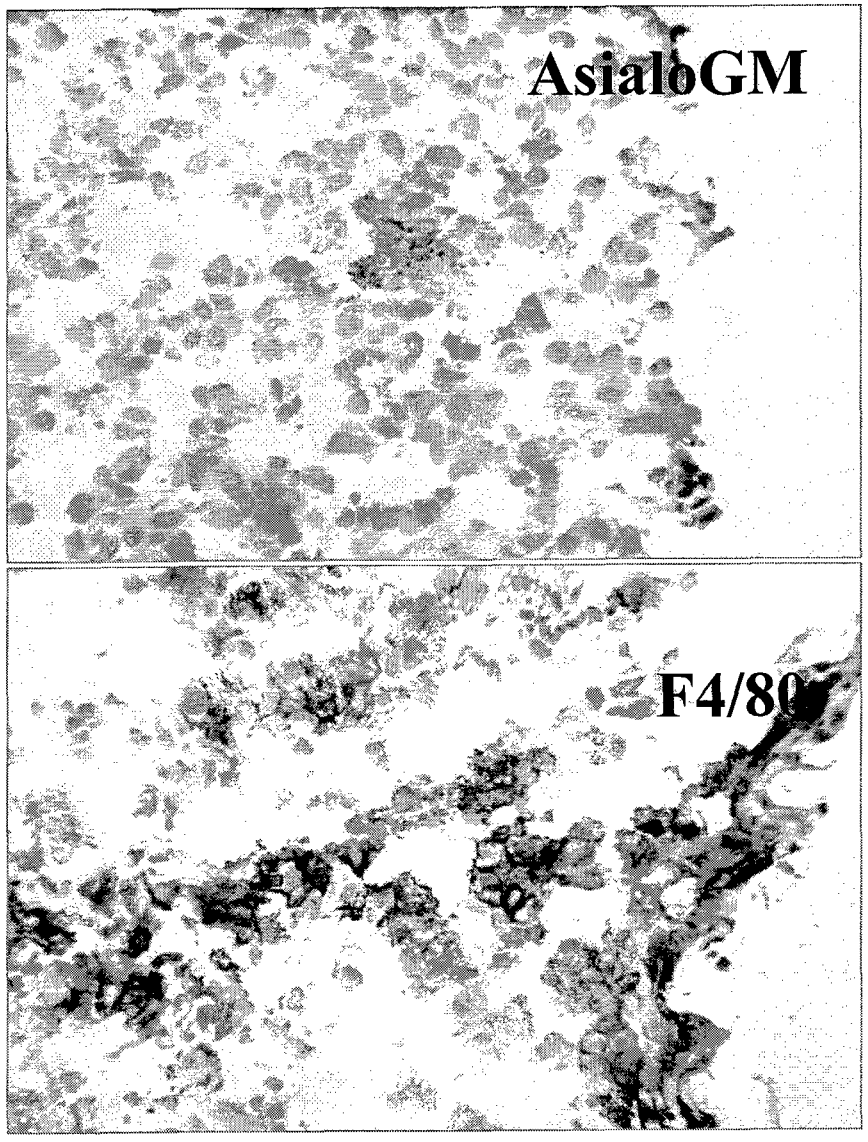


**RM1LacZ/IL12/IL18/saline**



**RM1CDUPRT/IL12/IL18/5FC**

Figure 8: Asialo GM+ cells that include NK cells were present in RM1 prostate cancers harvested after various treatments as shown.




Mouse #96.2
RM1CDUPRT/IL12/IL18/5FC

Mouse #96.3
RM1CDUPRT/IL12/IL18/saline

Figure 9: AsialoGM and F4/80 cells were present in higher numbers in RM1CDUPRT prostate tumors treated with both mIL12 + mIL18 in mice treated with 5FC compared with saline suggesting immunosuppressive effect of 5FU generated by GDEPT.

**Appendix 3: Abstracts presented.**

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American Association for Cancer Research

Proceedings of the 96th American Association for Cancer Research, Anneheim, April, 2005

**Control/Tracking Number :** 05-AB-5174-AACR
**Activity :**Abstract Submission
**Current Date/Time :** 11/28/2004 11:32:47 PM

**Gene directed enzyme prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer.**

**Short Title:**
CDUPRT-GDEPT for prostate cancer

*Pamela J. Russell, Aparajita Khatri, Bing Zhang, Eboney Doherty, Kim Ow, Jane Chapman, Rosetta Martiniello-Wilks*. Prince of Wales Hospital, Randwick, Australia, Prince of Wales Hospital, Sydney, Australia, Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using the fusion gene,cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) for treating prostate cancer (PCa).
*Objective*: To test the efficacy of CDUPRT-GDEPT against RM1 mouse androgen-refractory PCa grown in C57BL/6 mice: RM1 cells were stably transfected with green fluorescence protein (GFP) and the fusion gene, CDUPRT, derived from *E coli* (RM1-GFP/CDUPRT). **CD/UPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, thus killing both dividing and non-dividing cells.** This is especially relevant to PCa, which is characterized by a low proportion of dividing cells.
***Experimental Design***: RM1 cells were stably transfected with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ (controls) using lipofectamine. Cells that highly expressed GFP were selected by flow cytometry and used for further study. Transgene CDUPRT expression in cell lysates from cells grown *in vitro* or after *in vivo* implantation of RM1-GFP/CDUPRT was assessed by enzymic conversion of its substrate using HPLC. To assess the local bystander effect of CDUPRT-GDEPT, C57BL/6 mice were implanted directly into the prostate with cell mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions; 4 days later, 5FC was given intraperitoneally (ip) for 13 days at 500mg/kg/mouse/day. Pseudo-metastases in the lungs were established by a tail vein injection (iv) of untransfected RM1 cells 4 days post intraprostatic implantation. Mice were euthanased on day 19, and prostate weight and volume, and lung weight and colony counts were assessed. Tumors, lymph nodes, spleens and lungs were frozen or fixed for immunohistochemistry.
***Results***: Intraprostatic RM1-GFP/CDUPRT tumors on treatment with 5FC for 13 days resulted in complete regression of the tumors. Injection of cell mixtures (RM1-GFP/CDUPRT + RM1-GFP) resulted in a local bystander effect when only 20% of the cells were expressing the CDUPRT transgene. Interestingly, the lung colony counts indicated the presence of a distant bystander effect. The pseudo-metastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group

compared with the control groups. This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.

*Conclusions and future work*: The CDUPRT GDEPT leads to a significant local and a distant bystander effect when used to treat androgen refractory RM1 tumors in mice. The role of the immune system in this distant bystander effect is currently under investigation.

2. Australasian Gene Therapy Society Annual Meeting, Melbourne, 2005

**Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate cancer in C57BL/6 mice.**

*Aparajita Khatri*[1,2], *Pamela J. Russell*[1,2], *Bing Zhang*[3], *Eboney Doherty*[1,2], *Kim Ow*[1], *Jane Chapman*[1,2], *Rosetta Martiniello-Wilks*[4].

[1]Prince of Wales Hospital, Randwick, Australia,[2] University Of New South Wales, Sydney, [3]University of Queensland, Brisbane, [4]Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase (CD) in combination with uracil phosphoribosyl transferase (UPRT) for treating prostate cancer (PCa). *CDUPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites, including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, resulting in the killing of both dividing and non-dividing cells.* This is especially relevant to slow growing PCa.
Andogen-independent mouse RM1 cells were stably transformed with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ genes (controls). CDUPRT expression in cell lysates from RM1-GFP/CDUPRT cells/tumors was confirmed by estimation of enzymic conversion of its substrate, 5FC to 5FU using HPLC. Treatment of C57BL/6 mice bearing intraprostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. Further, intraprostatic implantations with mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions in C57BL/6 mice resulted in a ***'local bystander effect'***, even though only 20% of the cells were expressing the transgene. To determine if there was any distant bystander effect, pseudometastases in the lungs were established and the lung colony counts at necroscopy (day 19) indicated the presence of a ***'distant bystander effect'***. Indeed, the pseudometastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. ***This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.*** Furthermore, immunohistochemical evaluation of the GDEPT showed an increase in immune cell infiltration by $CD4^+$ T cells, macrophages and natural killer cells. There was increased tumor necrosis and apoptosis and a decrease in tumor vascularity after GDEPT. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate tumors via mechanisms involving necrosis and apoptosis, accompanied by strong infiltration of immune cells in the prostate tumors. The latter may be associated with the decrease in lung pseudometastases.